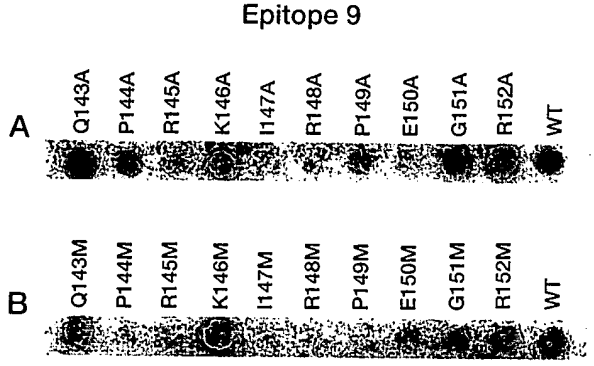


FIG. 1. **Single amino acid changes to epitope 9 result in loss of IgE binding to this epitope.** Epitope 9 was synthesized with an alanine (*Panel A*) or methionine (*Panel B*) residue substituted for one of the amino acids and probed with a pool of serum IgE from 15 patients with documented peanut hypersensitivity. The *letters* across the *top* of each *panel* indicate the one-letter amino acid code for the residue normally at the position and the amino acid that was substituted for this residue. The *numbers* indicate the position of each residue in the Ara h1 protein. *WT*, indicates the wild type peptide (no amino acid substitutions).

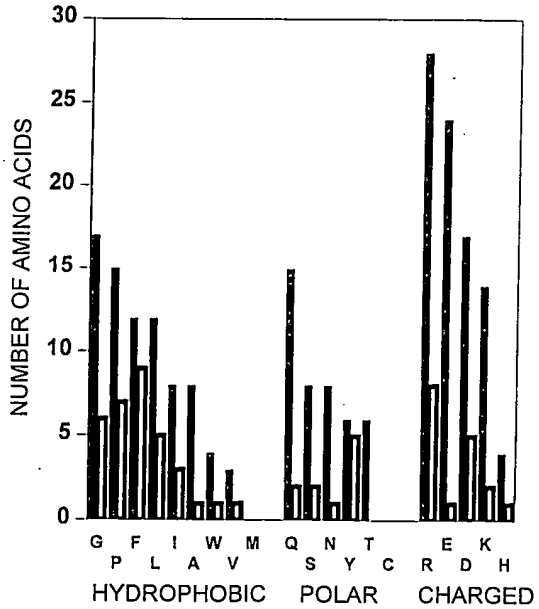


FIG. 2. **Hydrophobic amino acids are more critical to IgE binding.** The type of each amino acid within the Ara h1 epitopes was assessed relative to its importance to IgE binding. The *closed boxes* represent the total number of a particular type of amino acid residue found in all of the Ara h1 epitopes, whereas the *open boxes* represent the number of that type of residue which, when replaced, was found to result in the loss of IgE binding.

A

```
NNPFYFPSRR FSTRYGNQNGRIRVLQRFDQRSRQFQNLQNHRIVQIEAKPNTLVLP 227
DNPFYFNSDNSWNTLFKNQYGHIRVLQRFDQQSKRLQNLEDYRLVEFRSKPETLLLP

KHADADNILVIQQGQATVTVANGN NRKSFNLDEGH      ALRIPSGFISYILNRH 278
QQADAELLLVVRSGSAILVLVKPDDRREYFFLTSDNPIFSDHQKIPAGTIFYLVNPD

DNQNLRVAKISMPVNTPGQFEDFFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRV 335
PKEDLRIIQLAMPVNNPQIH EFFLSSTEAQQSYLQEFSKHILEASFNSKFEEINRV

LLEENAGGEQEERGQRRWSTRSSENNEGVIVKVSKEHVEELTKHAKSVSKKGSEEE 391
LFEEEGQQEGV                IVNIDSEQIKELSKHAKSSSRKSLSKQD

GDITNPINLREGEPDLSNNFGKLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHF 448
NTIGNEF          GNLTERTDNSLN      VLISSIEMEEGALFVPHY

NSKAMVIVVVNKGTGNLELVAVRKEQQQRGRREEEEDEDEEEEGSNREVRRYTARLK 505
YSKAIVILVVNEGEAHVELVGPKGNKETLEYE                SYRAELS

EGDVFIMPAAHPVAINASSELHLLGFGINAENNHRIFLAGDKDNVIDQIE     KQ 557
KDDVFVIPAAYPVAIKATSNVNFTGFGINANNNNRNLLAGKTDNVISSIGRALDGKD

AKDLAFPGSGEQVEKLIKNQKESHFVSAR 586
VLGLTFSGSGDEVMKLINKQSGSYFVDAH
```

B

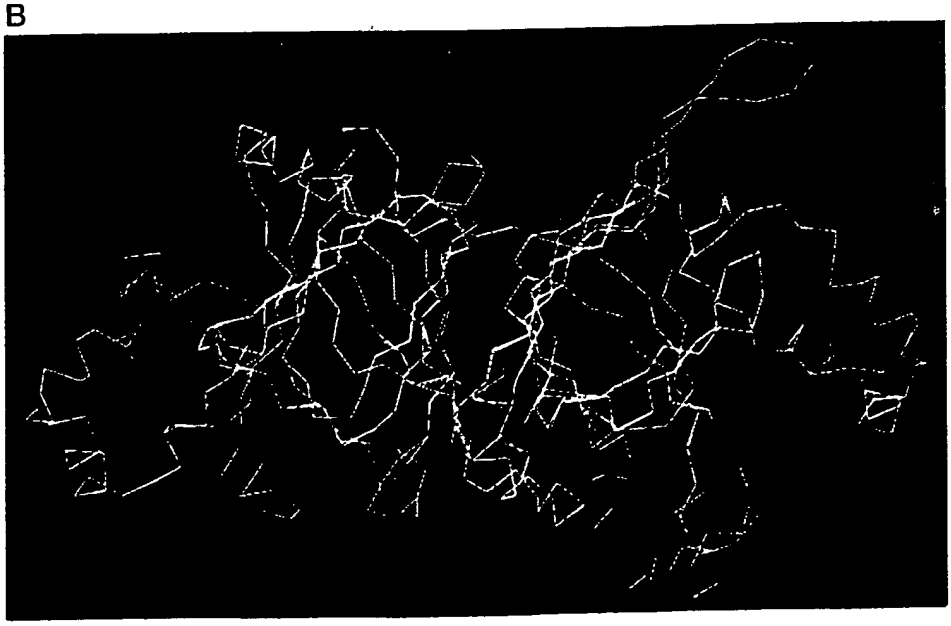


FIG. 3. **Alignment of the primary amino acid sequences and the α-carbon structural alignment of Ara h1 and the phaseolin A chain.** *Panel A* represents the single letter amino acid code for Ara h1 residues 172–586 (*top line*) and all of the phaseolin A chain (*bottom line*). The structurally conserved regions, shown in *bold type*, were used to develop the initial backbone of the Ara h1 model. The other regions were used in protein loop searches to complete the tertiary structure of Ara h1. *Panel B* represents the α-carbon alignment of the final model of Ara h1 (*white*) *versus* the phaseolin A chain (*yellow*). Labeled residues Asn^{1} and Arg^{415} represent the N and C termini of the Ara h1 model, respectively. Areas between labeled amino acids Asn^{169}-Val^{193}, Val^{212}-Gly^{221}, Phe^{240}-Pro^{226}, Pro^{226}-Phe^{240} and Arg^{300}-Asn^{323} represent areas of structural uncertainty due to insertions in Ara h1 or unsolved sequences in phaseolin. Note that the residue numbers are shifted due to the N-terminal deletion from the Ara h1 coding sequence found in the GenBank™ data bank (the amino acid sequence of this protein can be accessed through the GenBank™ data bank under GenBank Accession Number L34402 (17)).

Phi/Psi Plot

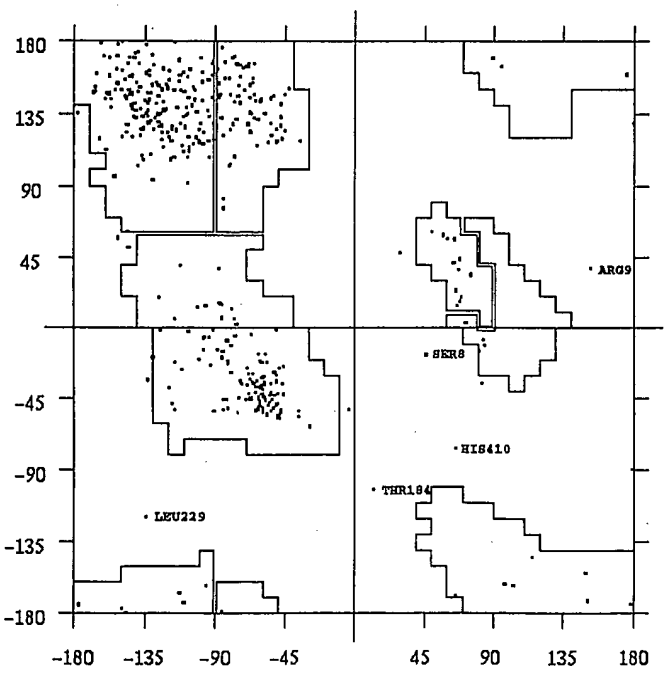


FIG. 4. **Most of the ϕ/ψ torsion angles of the amino acid residues in the Ara h1 tertairy structure model are allowed.** A plot of the ϕ and ψ angles for the amino acids in the Ara h1 tertiary structure model is shown. Each *dot within* one of the *boxes* represents an amino acid that has acceptable torsion angles. Major outliers are indicated by their three letter amino acid code and position using the N-terminal as residue 1 as in Fig. 3.

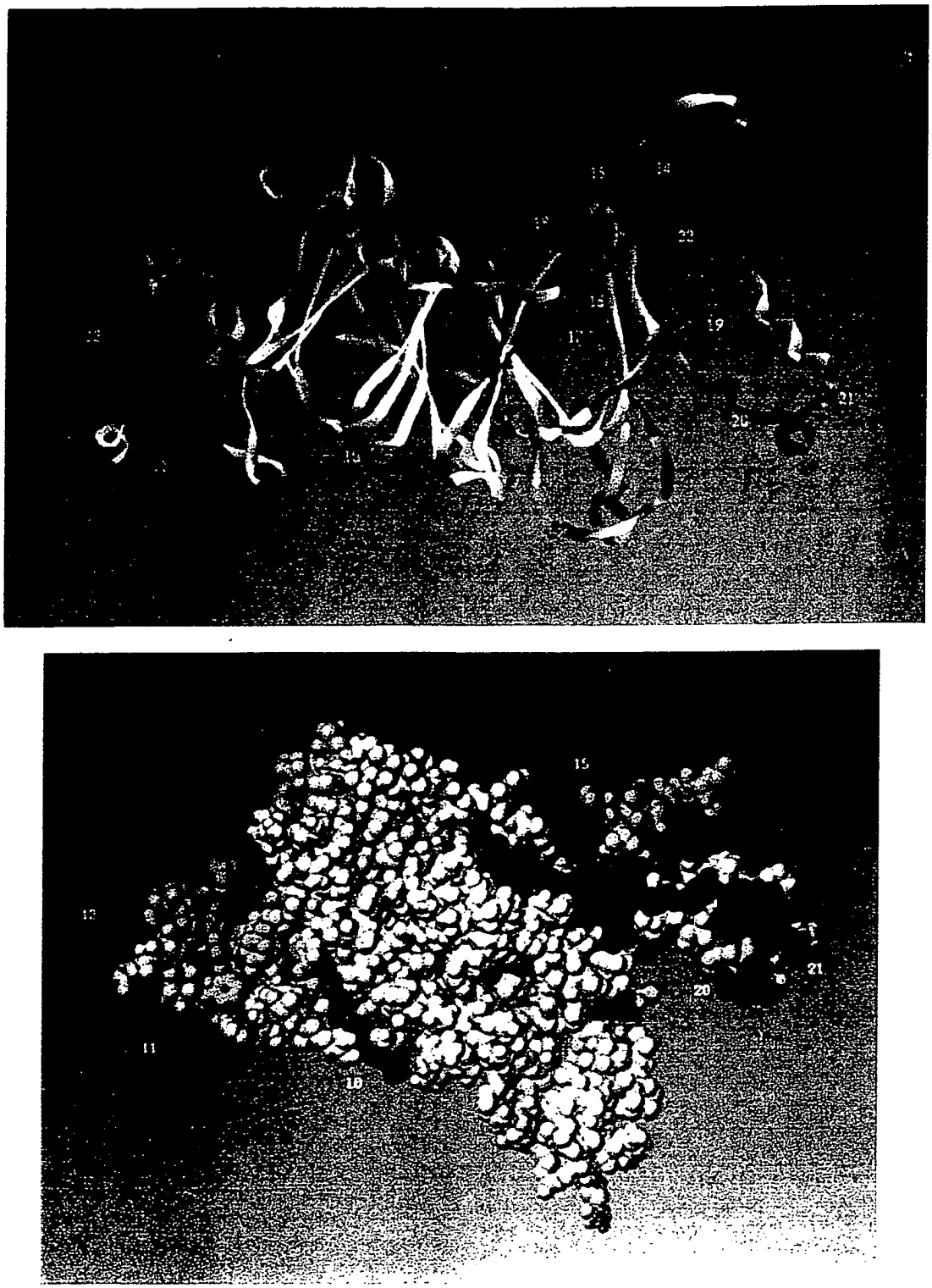


FIG. 5. **The majority of the Ara h1 IgE binding epitopes are clustered in two regions of the allergen.** The *top panel* represents a ribbon diagram of Ara h1 tertiary structure. The *numbered red areas* are IgE binding epitopes 10–22. Epitopes 13 and portions of 14 and 15 lie in an area of structural uncertainty. The *bottom panel* is a space filling model of Ara h1 tertiary structure. The *red* areas represent the IgE binding epitopes, and the *yellow atoms* are the residues that were determined to be critical for IgE binding to occur.

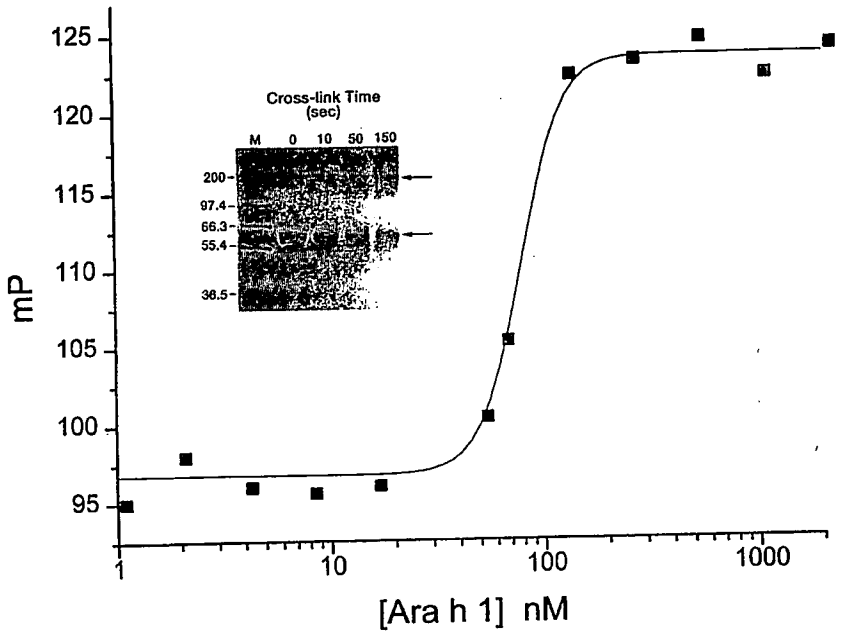


FIG. 6. **The Ara h1 allergen forms a stable trimeric structure.** Trace fluorescein-labeled Ara h1 was mixed with unlabeled Ara h1, and fluorescence polarization measurements (*mP*) were made at each concentration. Each point represents the average of three different experiments. Samples from the 200 nM concentration were then subjected to cross-linking with constant amounts of DSP for varying lengths of time, and the products were electrophoresed on SDS-polyacrylamide gels. Protein bands were visualized by Coomassie staining. *Lower arrow* indicates the Ara h1 monomer (~60 kDa), and the *upper band* represents the Ara h1 trimer (~180 kDa).

CHYMOTRYPSIN

Panel. A. **Panel. B.**

0 → 3 hrs 0 → 3 hrs

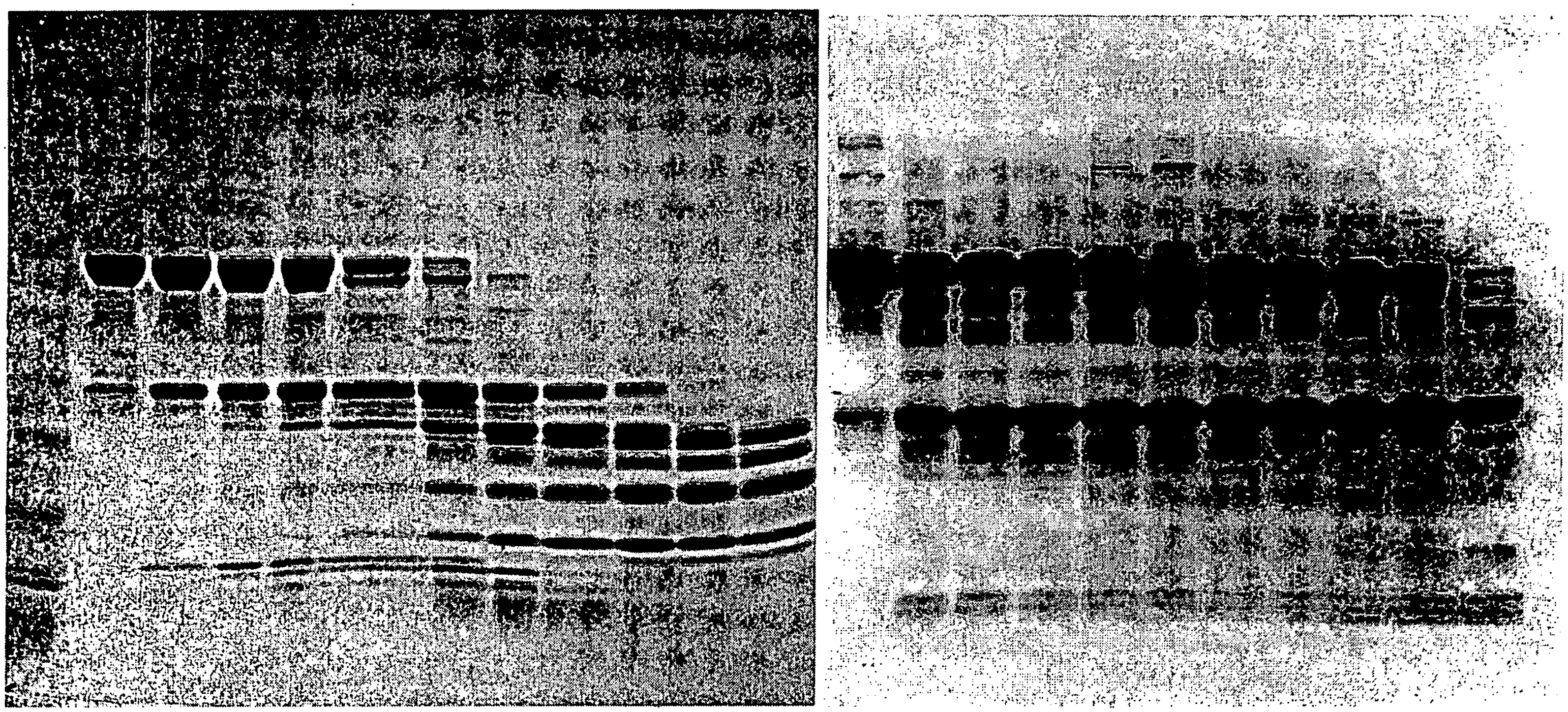


Fig. 7 (A, B)

CHYMOTRYPSIN

Panel C.

0 ⟶ 3 hrs

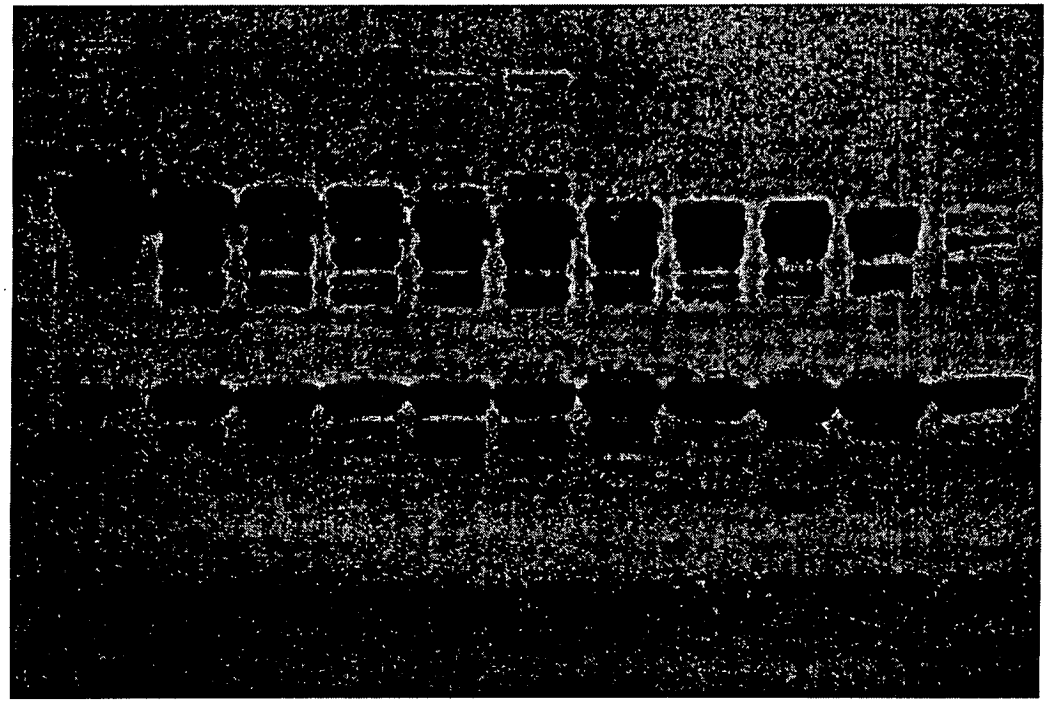
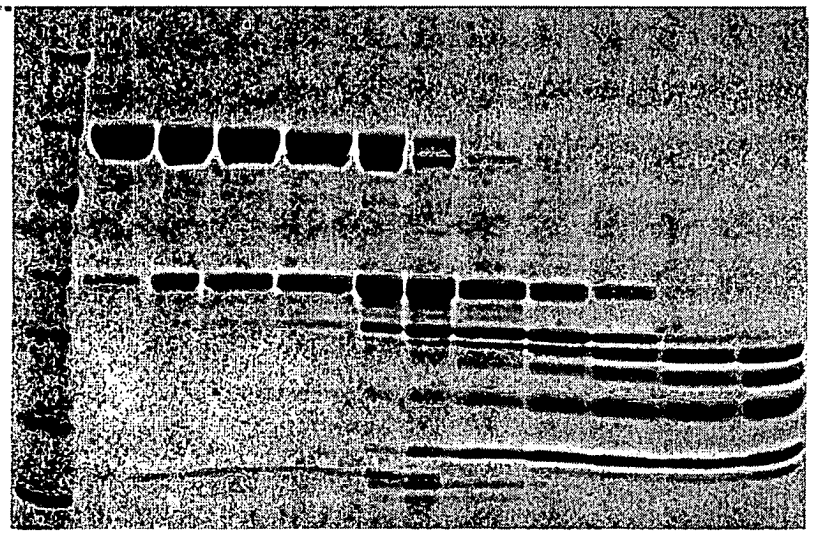


Fig. 7 (c)

TRYPSIN

Panel A.

0 → 3 hrs



Panel B.

0 → 3 hrs

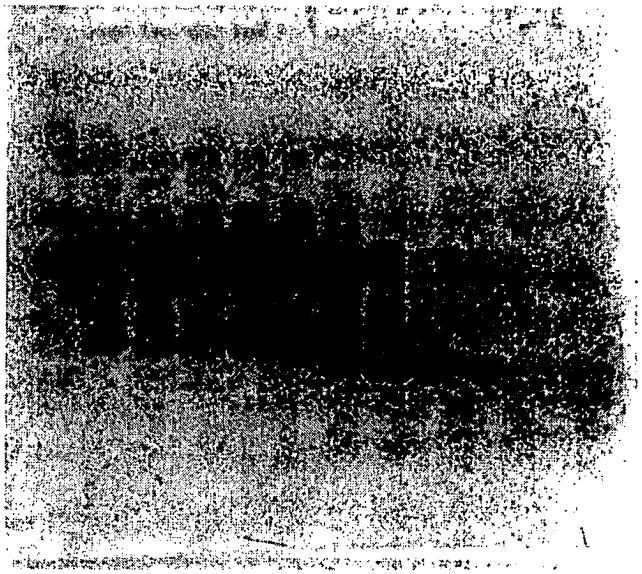


Fig. 8 (A, B)

TRYPSIN

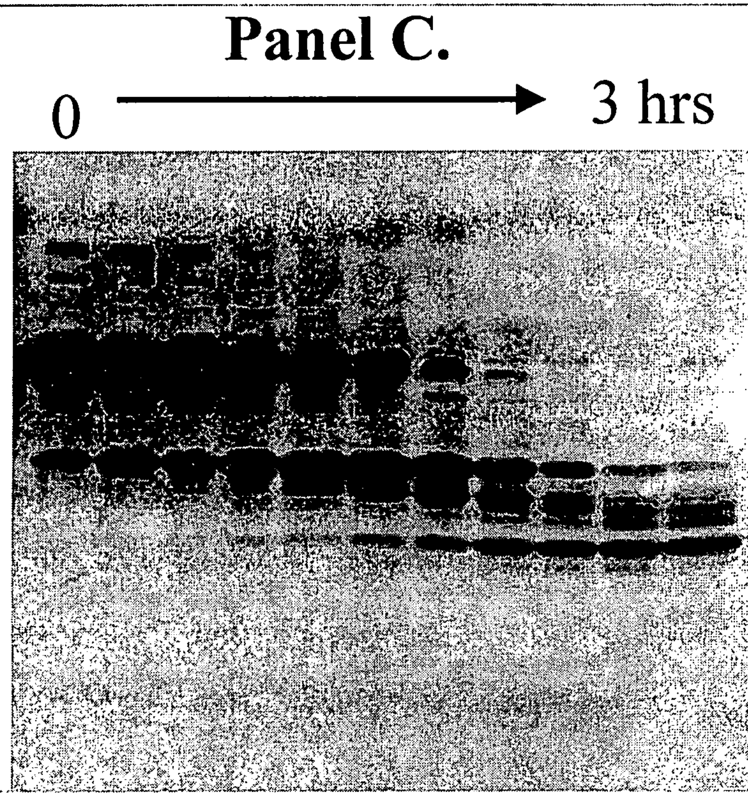


Fig. 8 (c)

Pepsin

Panel A 0 → 3 hrs

Panel B 0 → 3 hrs

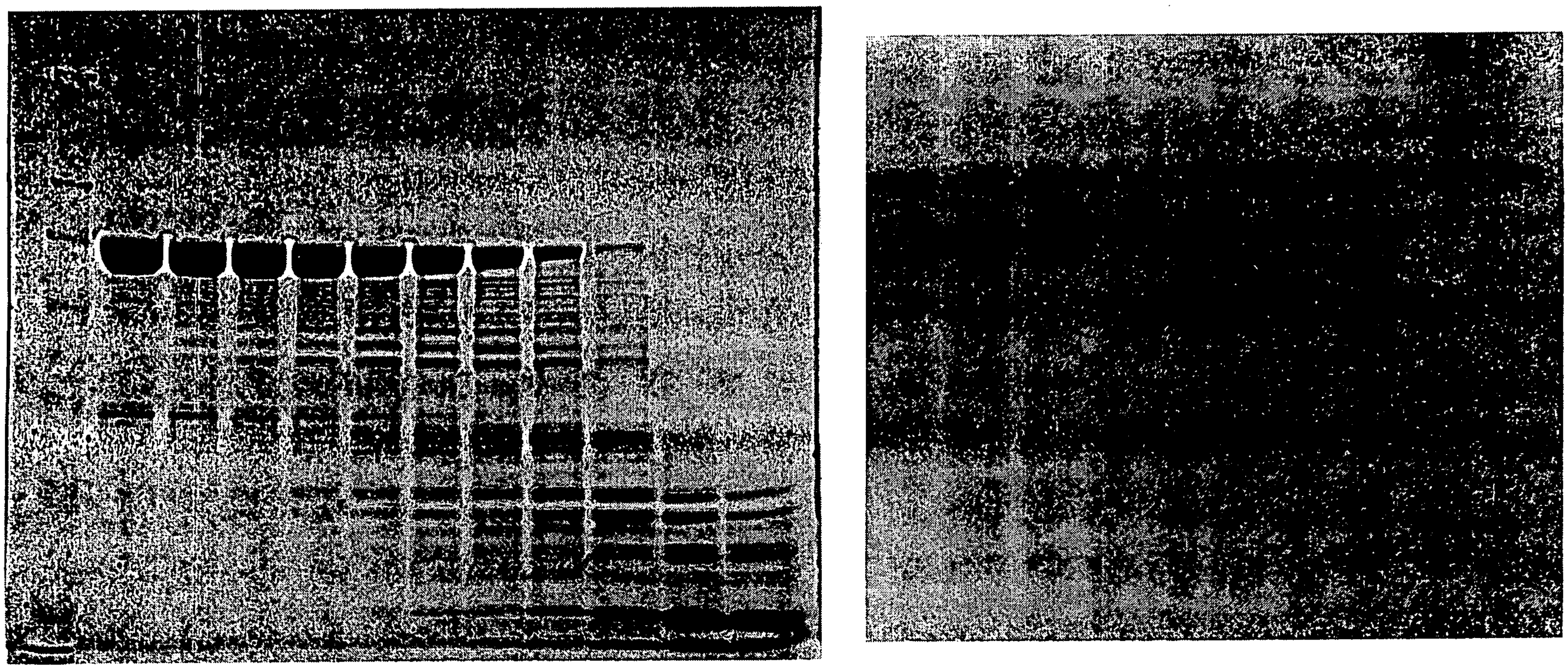


Fig. 9

MyoD Digestion

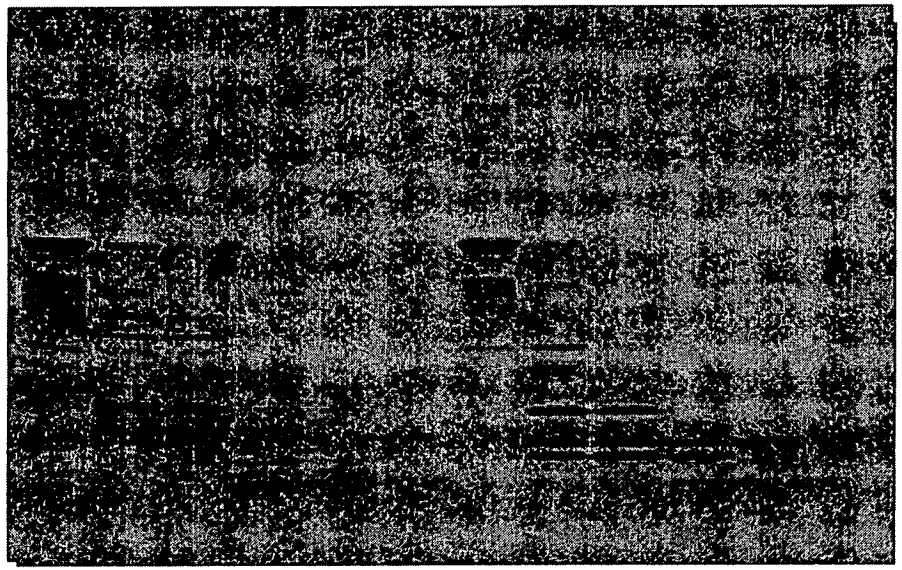


Fig. 10

Ara h 3 Amino Acid Sequence

1	I	S	F	R	Q	Q	P	E	E	N	A	C	Q	F	Q	R	L	N	A	Q	R	P	D	N	R	I	E
28	S	E	G	G	Y	I	E	T	W	N	P	N	N	Q	E	F	E	C	A	G	V	A	L	S	R	L	V
54	L	R	R	N	A	L	R	R	P	F	Y	S	N	A	P	Q	E	I	F	I	Q	Q	G	R	G	Y	F
82	G	L	I	F	P	G	C	P	R	H	Y	E	E	P	H	T	Q	G	R	R	S	Q	S	Q	R	P	P
109	R	R	L	Q	G	E	D	Q	S	Q	Q	Q	R	D	S	H	Q	K	V	H	R	F	D	E	G	D	L
136	I	A	V	P	T	G	V	A	F	W	L	Y	N	D	H	D	T	D	V	V	A	V	S	L	T	D	T
163	N	N	N	D	N	Q	L	D	Q	F	P	R	R	F	N	L	A	G	N	T	E	Q	E	F	E	F	L
190	R	Y	Q	Q	Q	S	R	Q	S	R	R	R	S	L	P	Y	S	P	Y	S	P	Q	S	Q	P	R	Q
207	E	E	R	E	F	S	P	R	G	Q	H	S	R	R	E	R	A	G	Q	E	E	E	N	E	G	G	N
234	I	F	S	G	F	T	P	E	F	L	E	Q	A	F	Q	V	D	D	R	Q	I	V	Q	N	L	R	G
261	E	T	E	S	E	E	E	G	A	I	V	T	V	R	G	G	L	R	I	L	S	P	D	R	K	R	R
288	A	D	E	E	E	E	Y	D	E	D	E	Y	E	Y	D	E	E	D	R	R	R	G	R	G	S	R	G
315	R	G	N	G	I	E	E	T	I	C	T	A	S	A	K	K	N	I	G	R	N	R	S	P	D	I	Y
342	N	P	Q	A	G	S	L	K	T	A	N	D	L	N	L	L	I	L	R	W	L	G	L	S	A	E	Y
369	G	N	L	Y	R	N	A	L	F	V	A	H	Y	N	T	N	A	H	S	I	I	Y	R	L	R	G	R
396	A	H	V	Q	V	V	D	S	N	G	N	R	V	Y	D	E	E	L	Q	E	G	H	V	L	V	V	P
423	Q	N	F	A	V	A	G	K	S	Q	S	E	N	F	E	Y	V	A	F	K	T	D	S	R	P	S	I
450	A	N	L	A	G	E	N	S	V	I	D	N	L	P	E	E	V	V	A	N	S	Y	G	L	Q	R	E
477	Q	A	R	Q	L	K	N	N	N	P	F	K	F	F	V	P	P	S	Q	Q	S	P	R	A	V	A	

Fig. 11

A.

```
            51                                                  100
Ara h 3   SEGGYIETWN PNNQEFECAG VALSRLVLRR NALRRPFYSN APQEIFIQQG
 G1 Soy   SEGGLIETWN PNNKPFQCAG VALSRCTLNR NALRRPSYTN GPQEIYIQQG
 G2 Soy   SEGGFIETWN PNNKPFQCAG VALSRCTLNR NALRRPSYTN GPQEIYIQQG
 A2 Pea   SEGGLIETWN PNNKQFRCAG VALSRATLQH NALRRPYYSN APQEIFIQQG

            101                                                 150
Ara h 3   RGYFGLIFPG CPRHYEEPHT QGRRSQSQRP PRRLQGEDQS QQQRDSHQKV
 G1 Soy   KGIFGMIYPG CPSTFEEPQQ PQQRGQSSRP .......... ...QDRHQKI
 G2 Soy   NGIFGMIFPG CPSTYQEPQE SQQRGRSQRP .......... ...QDRHQKV
 A2 Pea   NGYFGMVFPG CPETFEEPQE SEQ.GEGRRY .......... ...RDRHQKV
```

B.

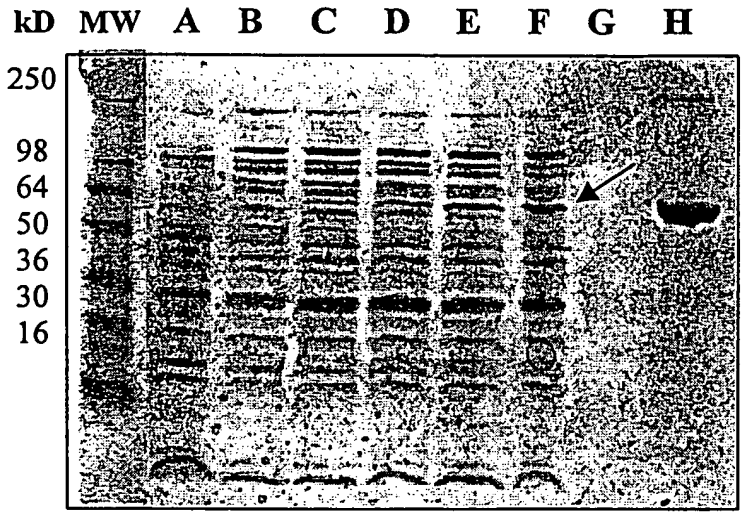
```
            351                                                 400
Ara h 3   EYDEDEY... .....EYDEE DRRRGRGSRG R......GNG IEETICTASA
 G1 Soy   EEEEDEKP.. ...QCKGKDK HCQRPRGSQS KSRR....NG IDETICTMRL
 G2 Soy   DDDEEEQP.. ...QCVETDK GCQR....QS KRSR....NG IDETICTMRL
 A2 Pea   DEDEERQPRH QRRRGEEEEE DKKERRGSQK GKSRRQGDNG LEETVCTAKL

            401                                                 450
Ara h 3   KKNIGRNRSP DIYNPQAGSL KTANDLNLLI LRWLGLSAEY GNLYRNALFV
 G1 Soy   RHNIGQTSSP DIYNPQAGSV TTATSLDFPA LSWLRLSAEF GSLRKNAMFV
 G2 Soy   RQNIGQNSSP DIYNPQAGSI TTATSLDFPA LWLLKLSAQY GSLRKNAMFV
 A2 Pea   RLNIGPSSSP DIYNPEAGRI KTVTSLDLPV LRWLKLSAEH GSLHKNAMFV

            451                                                 500
Ara h 3   AHYNTNAHSI IYRLRGRAHV QVVDSNGNRV YDEELQEGHV LVVPQNFAVA
 G1 Soy   PHYNLNANSI IYALNGRALI QVVNCNGERV FDGELQEGRV LIVPQNFVVA
 G2 Soy   PHYTLNANSI IYALNGRALV QVVNCNGERV FDGELQEGGV LIVPQNFAVA
 A2 Pea   PHYNLNANSI IYALKGRARL QVVNCNGNTV FDGELEAGRA LTVPQNYAVA
```

Fig. 12

A. *Bacterial Expression of Recombinant Ara h 3*



B. *Immunoblot Analysis of Total Bacterial Extract*

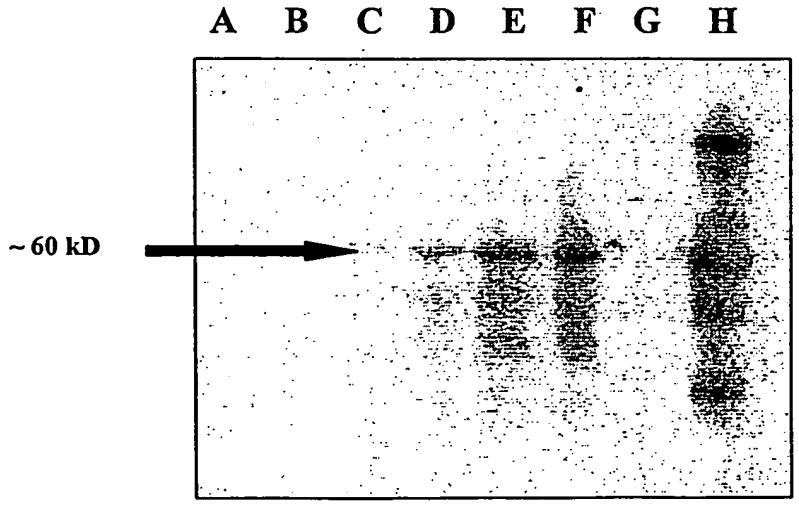
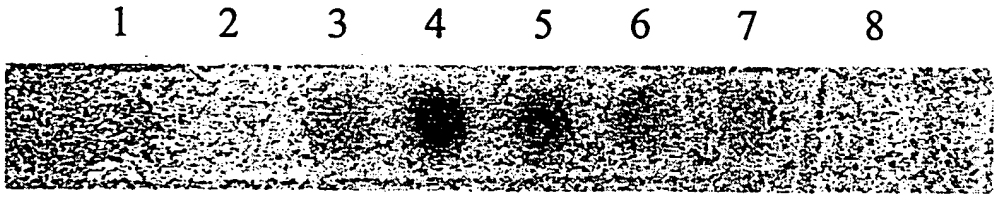


Fig. 13

A.

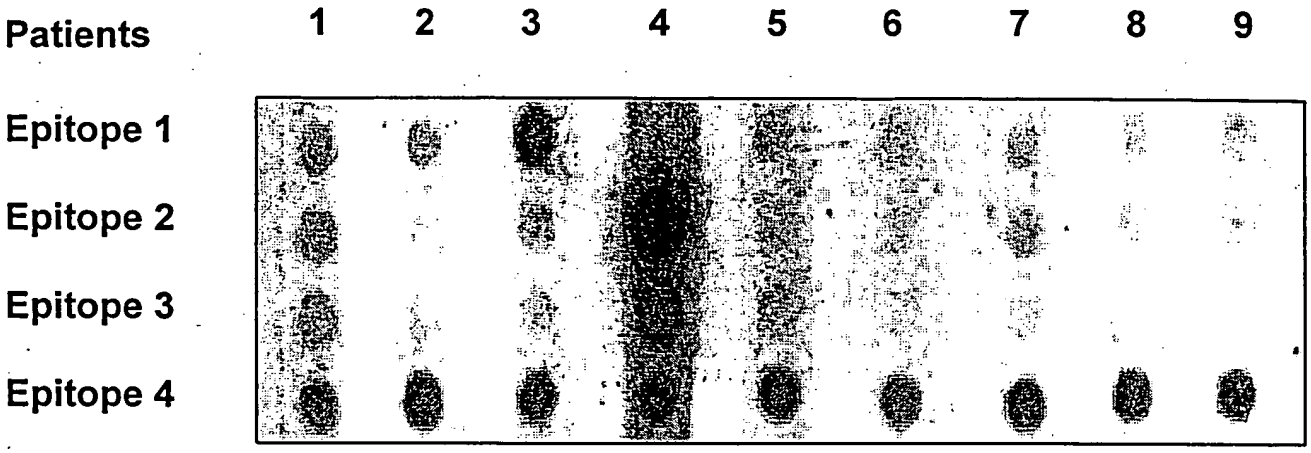


B.

```
   LSAEYGNLYRNALFVAHYNTNAHS
1. LSAEYGNLYR
2.   AEYGNLYRNA
3.     YGNLYRNALF
4.       NLYRNALFVA
5.         YRNALFVAHY
6.           NALFVAHYNT
7.             LFVAHYNTNA
8.               VAHYNTNAHS
```

Fig. 14

A. *Epitope 4 is an Immunodominant Epitope*



4

B. *Percentage of Recognition for Each Epitope*

Epitope	Sequence	Position	Percentage
1	EQEFLRYQQQ	183-192	5% (1/20)
2	FTPEFLEQAF	246-255	25% (5/20)
3	EYEYDEEDRR	300-309	35% (7/20)
4	LYRNALFVAH	379-388	100%(20/20)

Fig. 15

Peptide 2

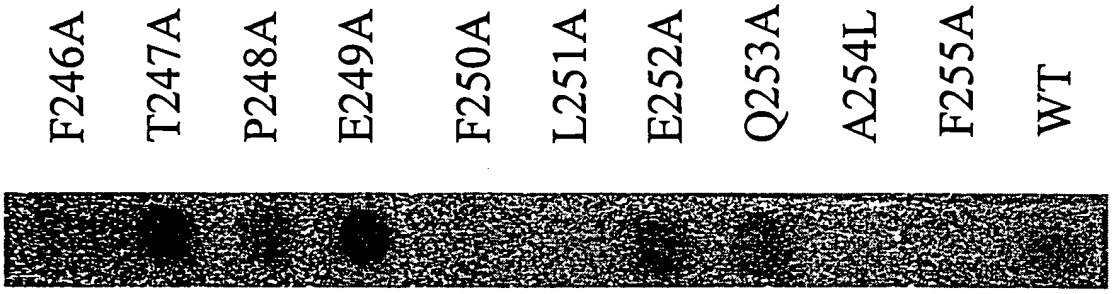
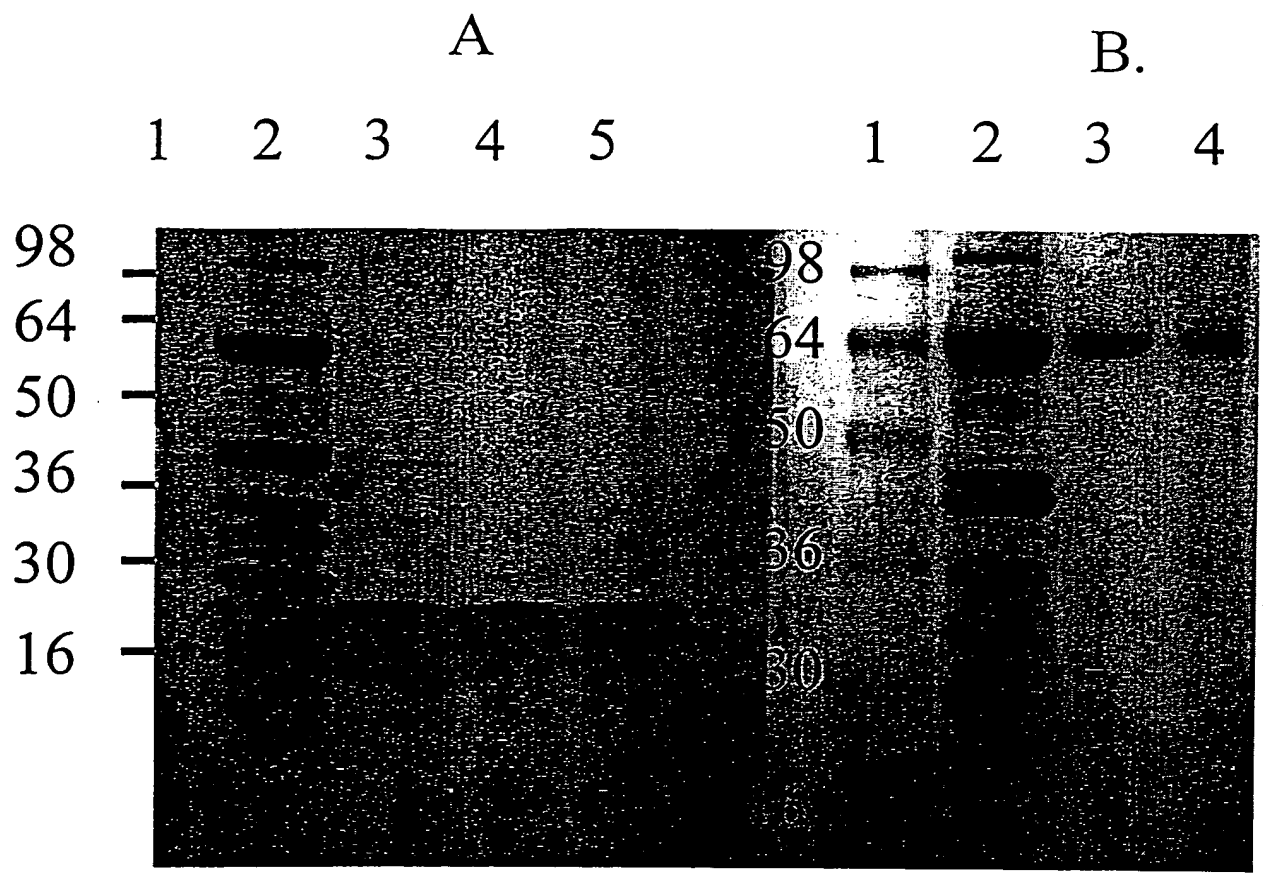


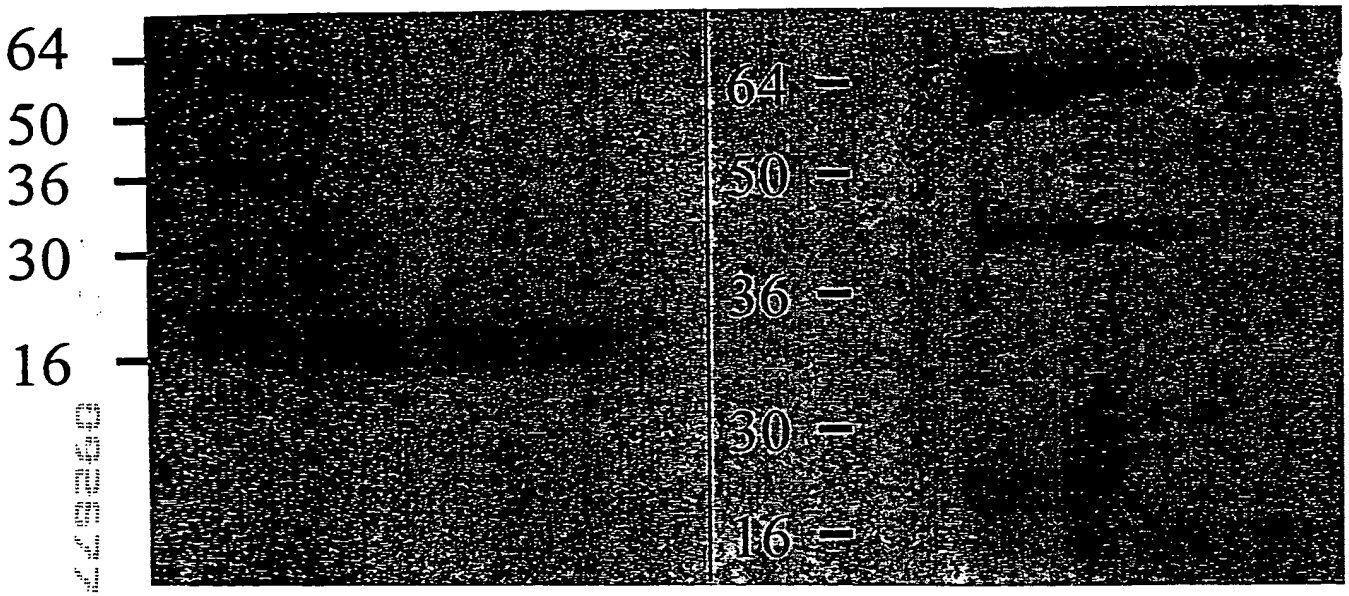
Fig. 16



SDS-PAGE analysis of the protein profiles at various stages of allergen purification.

Panels A and B are the protein profiles resulting from the purification of Ara h 1 and Ara h 2, respectively. Lanes are as follows: *lanes A1 and B1*, protein standards; *lanes A2 and* B2, crude peanut extract; lane *A3*, 25% ammonium sulfate pellet; *A4*, Ara h 2 fraction following anion exchange chromatography; *lane A5*, Ara h 2 fraction following hydrophobic chromatography *lane B3*, 100% ammonium sulfate pellet; *lane B4*, Ara h 1 fraction following cation exchange chromatography.

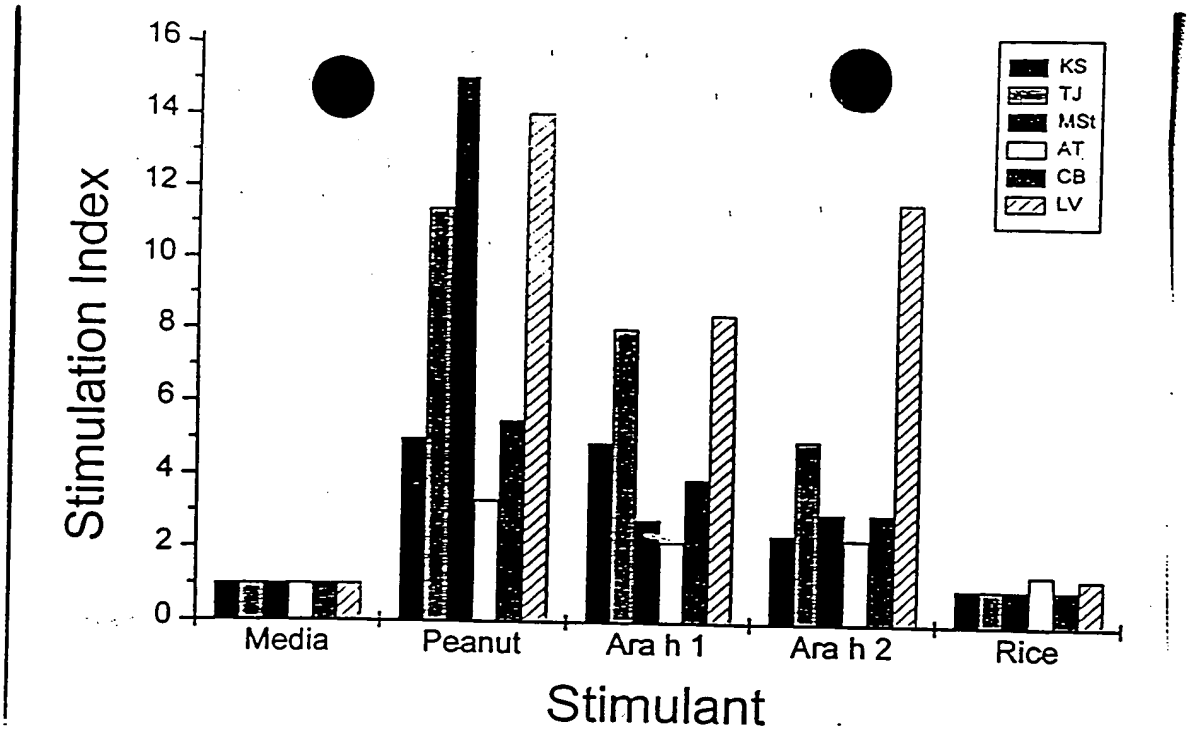
Fig. 17



Immunoblot of the purified proteins using serum IgE from allergic individuals.

Panels A and B are the protein profiles resulting from the purification of Ara h 1 and Ara h 2 that were blotted to nitrocellulose and detected by western blot analysis using serum IgE from allergic individuals as the primary antibody. Lanes are as *follows: lanes A1 and B1*, protein standards; *lanes A2 and* B2, crude peanut extract; lane *A3*, 25% ammonium sulfate pellet; *A4*, Ara h 2 fraction following anion exchange chromatography; *lane A5*, Ara h 2 fraction following hydrophobic chromatography *lane B3*, 100% ammonium sulfate pellet; *lane B4*, Ara h 1 fraction following cation exchange chromatography.

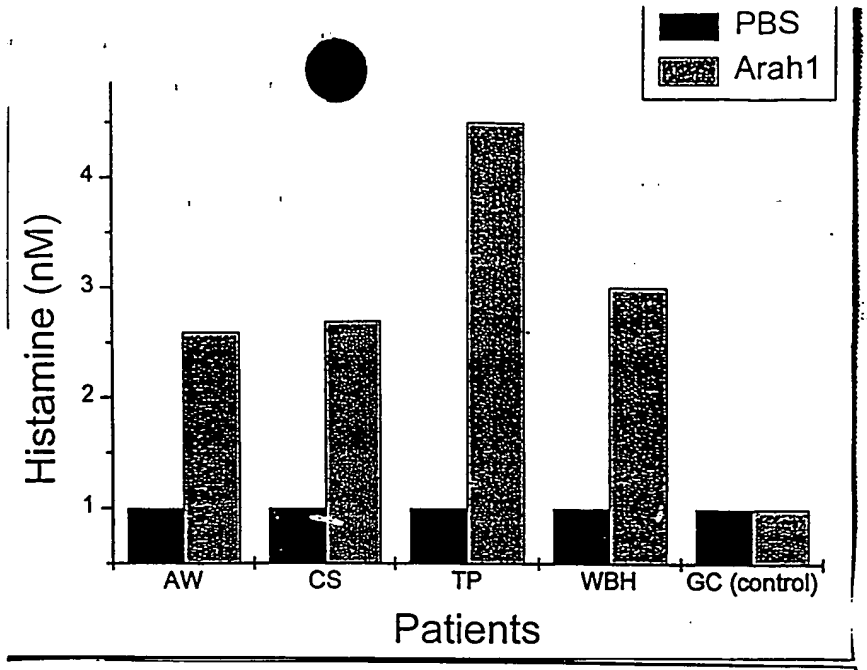
Fig. 18

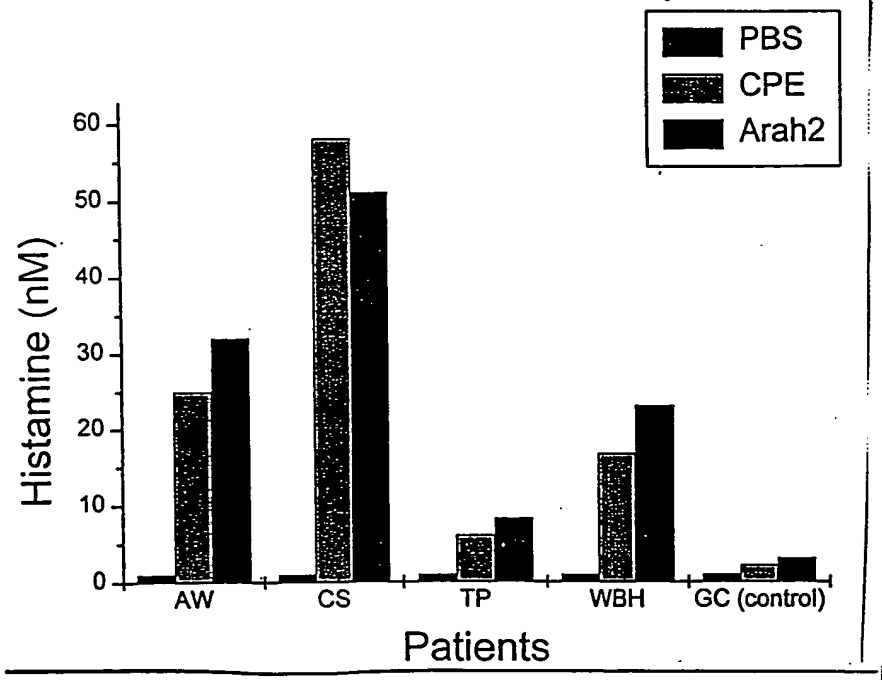


Purified Ara h 1 and Ara h 2 can stimulate T cell proliferation.

T cells were isolated from peanut allergic individuals and placed into 96 well plates at 4 x 10^4 cells/well and treated in triplicates with media, crude peanut extracts (positive control), Ara h 1, Ara h 2 or rice extracts (negative control). The cells were allowed to proliferate for 6 days and then incubated with ^{3}H-thymidine (1μCi/well) at 37 C for 6-8 hrs and then harvested onto glass fiber filters. T-cell proliferation was estimated by quantitating the amount of ^{3}H-thymidine incorporation into the DNA of proliferating cells. ^{3}H-thymidine incorporation is reported as stimulation (SI) above media treated control cells.

Fig. 19

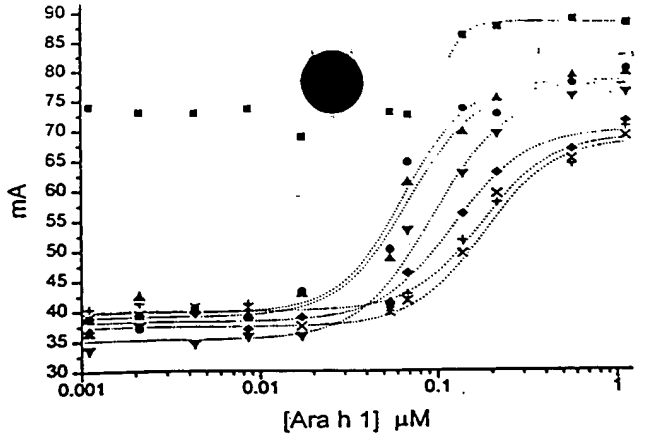




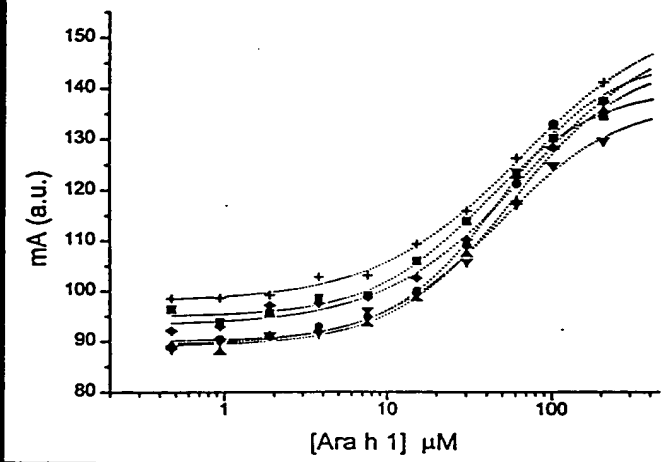
Purified Ara h 1 and Ara h 2 can stimulate histamine release from mast cells of peanut allergic individuals.

Mast cells from whole blood of allergic individuals (on the x-axis) from left to right were treated with PBS (negative control), crude peanut extracts (positive control), Ara h 1 (panel A) or Ara h 2 (panel B). The release of histamine is reported on the y-axis in nM. The histamine release assay was that developed by Immunologics (City, State) and it was performed exactly as described by the manufacturer.

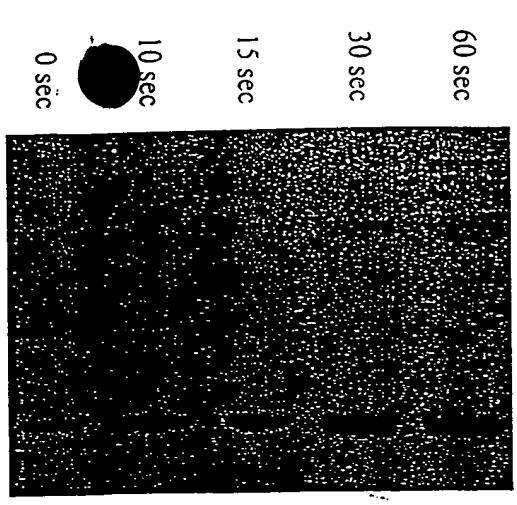
Fig. 20



Panel A: The formation of trimers at low concentrations of Ara h 1. The samples were in binding buffer plus various concentrations of NaCl as indicated: (○) 0 mM NaCl, (○) 100 mM NaCl, (·) 300 mM NaCl, (–) 500 mM NaCl, (–) 900 mM NaCl, (▯) 1400 mM NaCl, (▯) 1800 mM NaCl.



Panel B: The formation of hexamers at high concentrations of Ara h 1. The samples were in binding buffer plus various concentrations of NaCl as indicated: (○) 100 mM NaCl, (○) 400 mM NaCl, (·) 600 mM NaCl, (–) 800 mM NaCl, (–) 1100 mM NaCl, (▯) 1300 mM NaCl, (▯) 1800 mM NaCl.

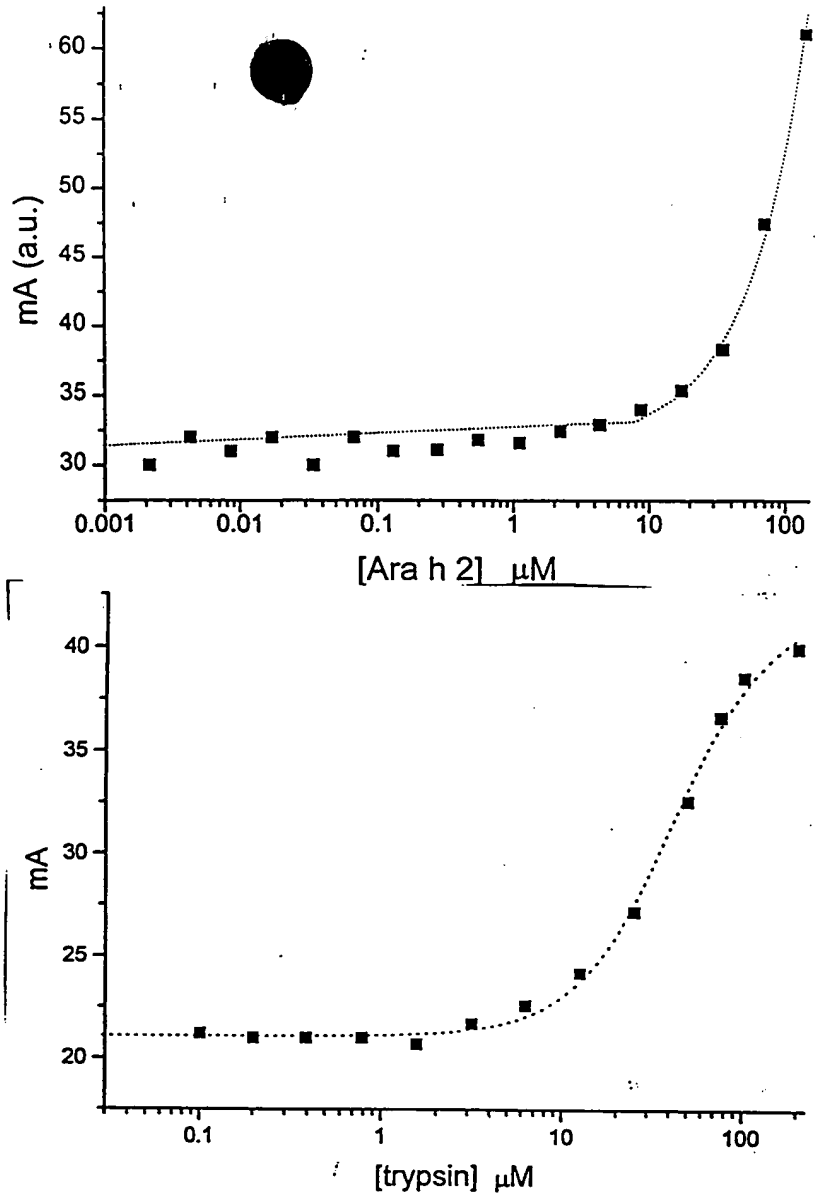


Panel C: Samples from the 80 µM concentration from Panel A were subjected to limited cross-linking at various time points and the products electrophoresed on SDS polyacrylamide gels. Protein bands were visualized by Coomassie staining. Lower arrow indicates the Ara h 1 monomer (~60 kDa), the next highest band represents the Ara h 1 trimer (~180 kDa), and the highest molecular weight band represents Ara h 1 hexamer (~360 kDa).

The purified Ara h 1 protein retains its native structure as indicated by its ability to form homotrimers and hexamers.

Fluorescence anisotropy was used to follow the formation of Ara h 1 higher order structure. All fluorescence measurements were made using a Beacon fluorescence polarization spectrometer (Pan Vera, Madison, WI) with fixed excitation (490 nm) and emission (530 nm) wavelengths which are specified for fluorescein use. Fluorescence measurements were done at room temperature (24 °C) in binding buffer (50 mM Tris, 1mM EDTA, 100mM NaCl, 2mM DTT, 5% glycerol, pH 7.5) in a final volume of 1.1 ml. A constant amount of fluorescein labeled protein (10 nM of Ara h 1) was diluted with binding buffer and mixed with various concentrations of unlabeled Ara h 1 to analyze homo-oligomer formation. Serial dilutions of the desalted proteins (by 0.5 or 0.8 increments) were made in binding buffer and the appropriate amounts were added to constant amounts of fluorescein-labeled protein. Each data point is an average of three independent measurements. The intensity of fluorescence remained constant throughout the anisotropy measurements.

Fig. 21



Ara h 2 does not form homo-oligomers but does bind to trypsin.

The fluorescence milli-anisotropy (mA) of fluorescent-labeled Ara h 2 titrated with unlabeled Ara h 2 or trypsin at different concentrations is measured and plotted. All fluorescent measurements were performed exactly as described in Fig. 5.

Panel A: **The fluorescence milli-anisotropy (mA) of fluorescent-labeled Ara h 2 titrated with unlabeled Ara h 2 at different concentrations is measured and plotted versus the concentration of unlabeled Ara h 2. The samples were in binding buffer plus ( )100 mM NaCl**

Panel B: **The fluorescence milli-anisotropy (mA) of fluorescent-labeled Ara h 2 titrated with unlabeled trypsin at different concentrations is measured and plotted versus the concentration of unlabeled trypsin. The samples were in binding buffer plus ( ) 100 mM NaCl.**

Fig. 22

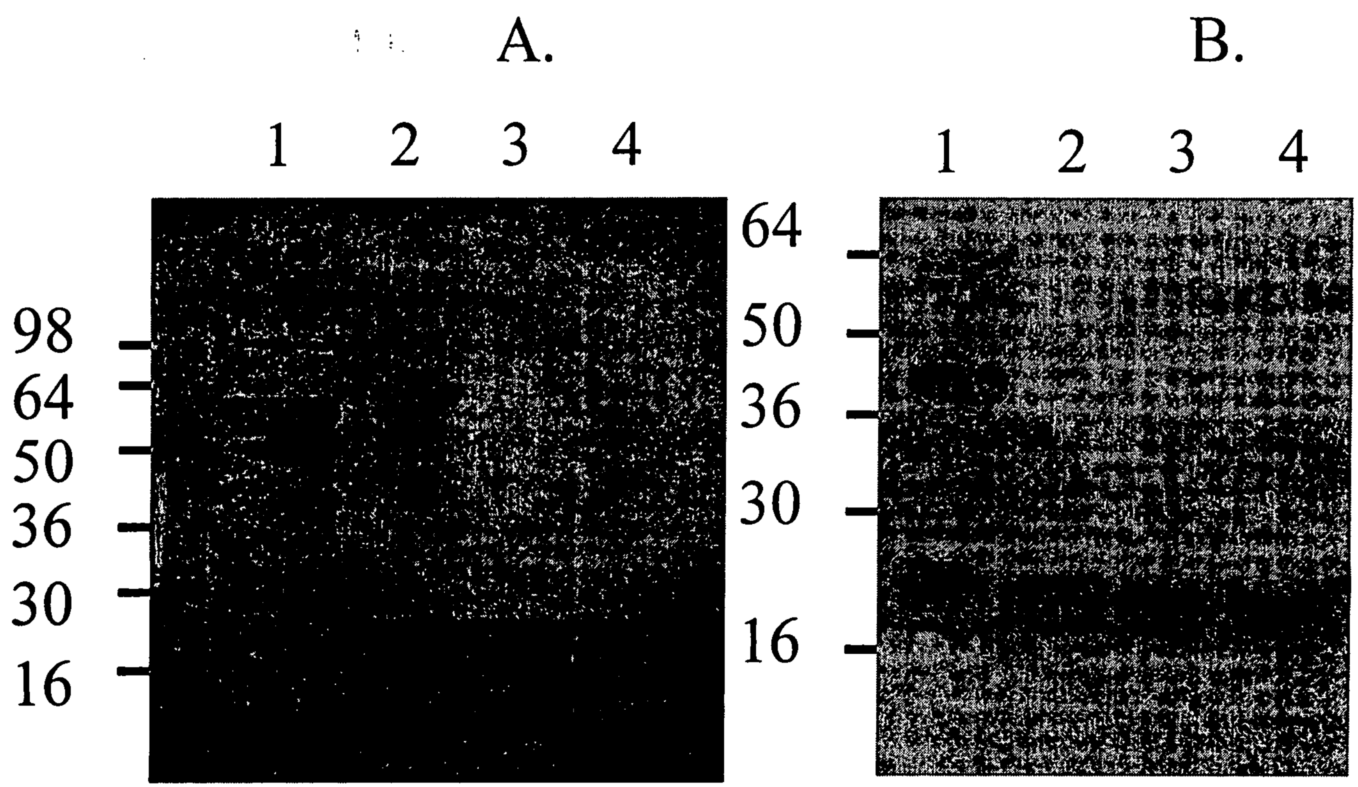


Fig. 23

Anti-Zap-70 Ip, Anti-TCR western

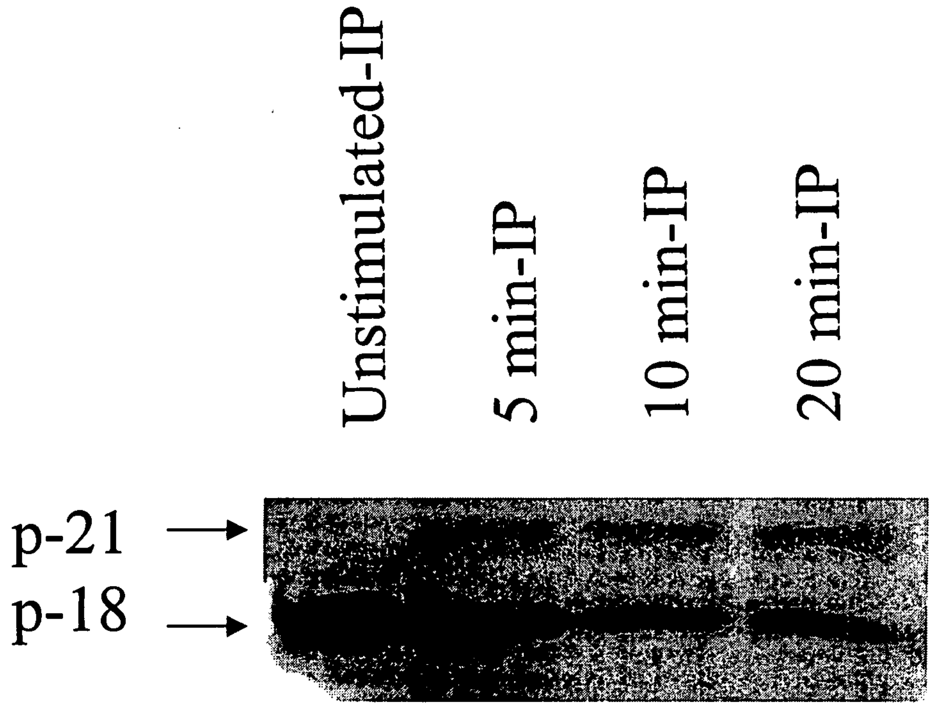


Fig. 24A

Anti-Zap-70-IP, Anti-pTyr western.

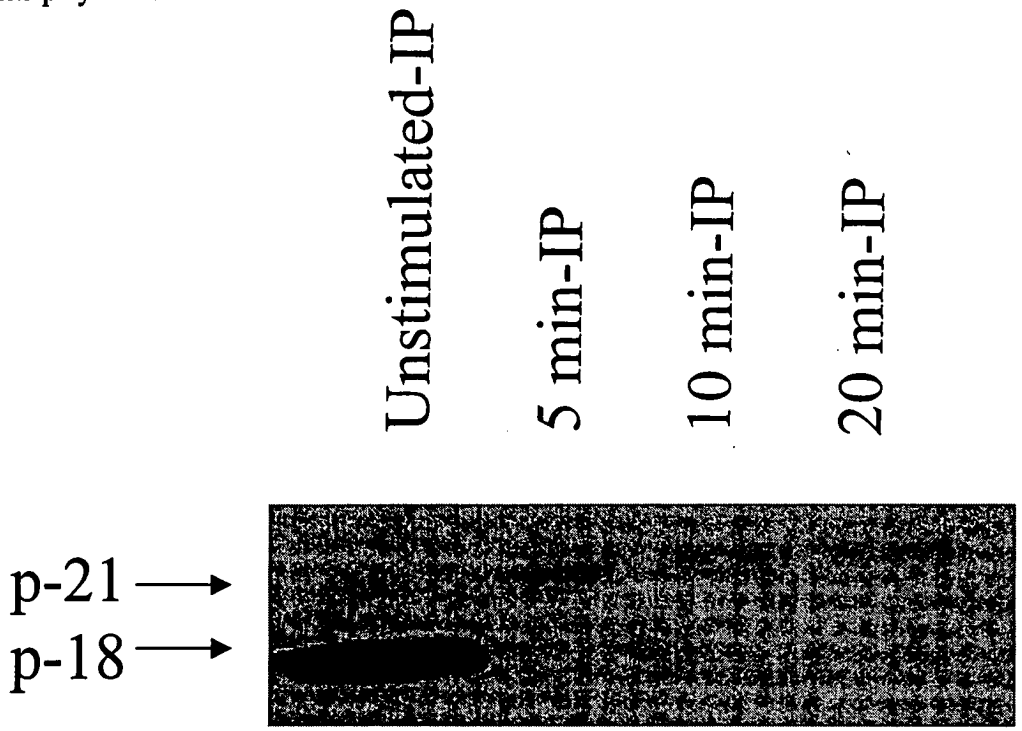


Fig. 24B

Anti-TCR-IP, Anti-ERK-1 western

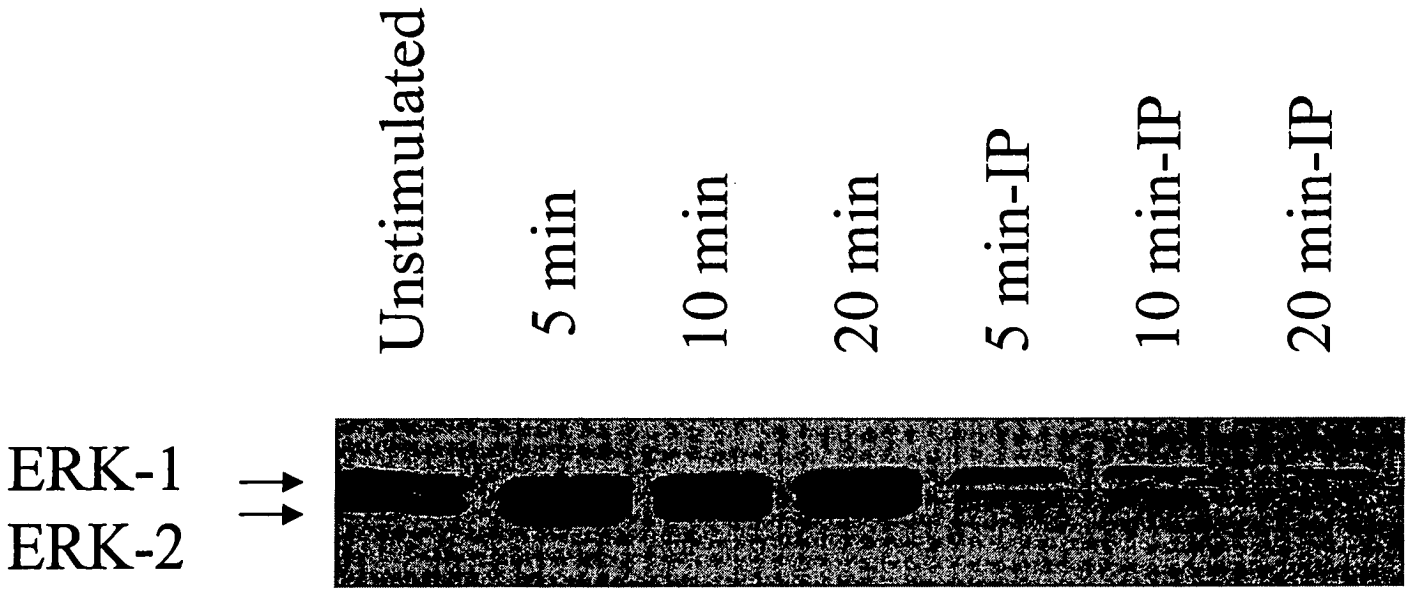


Fig. 24C

Anti-TCR-IP, Anti pTYR western.

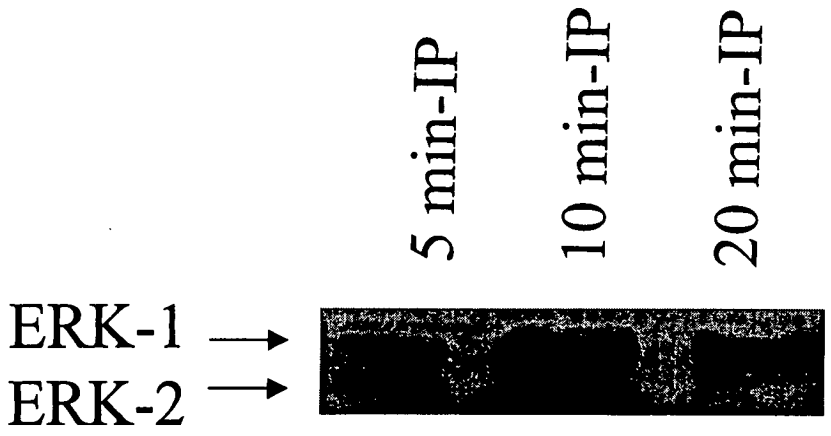


Fig. 24D

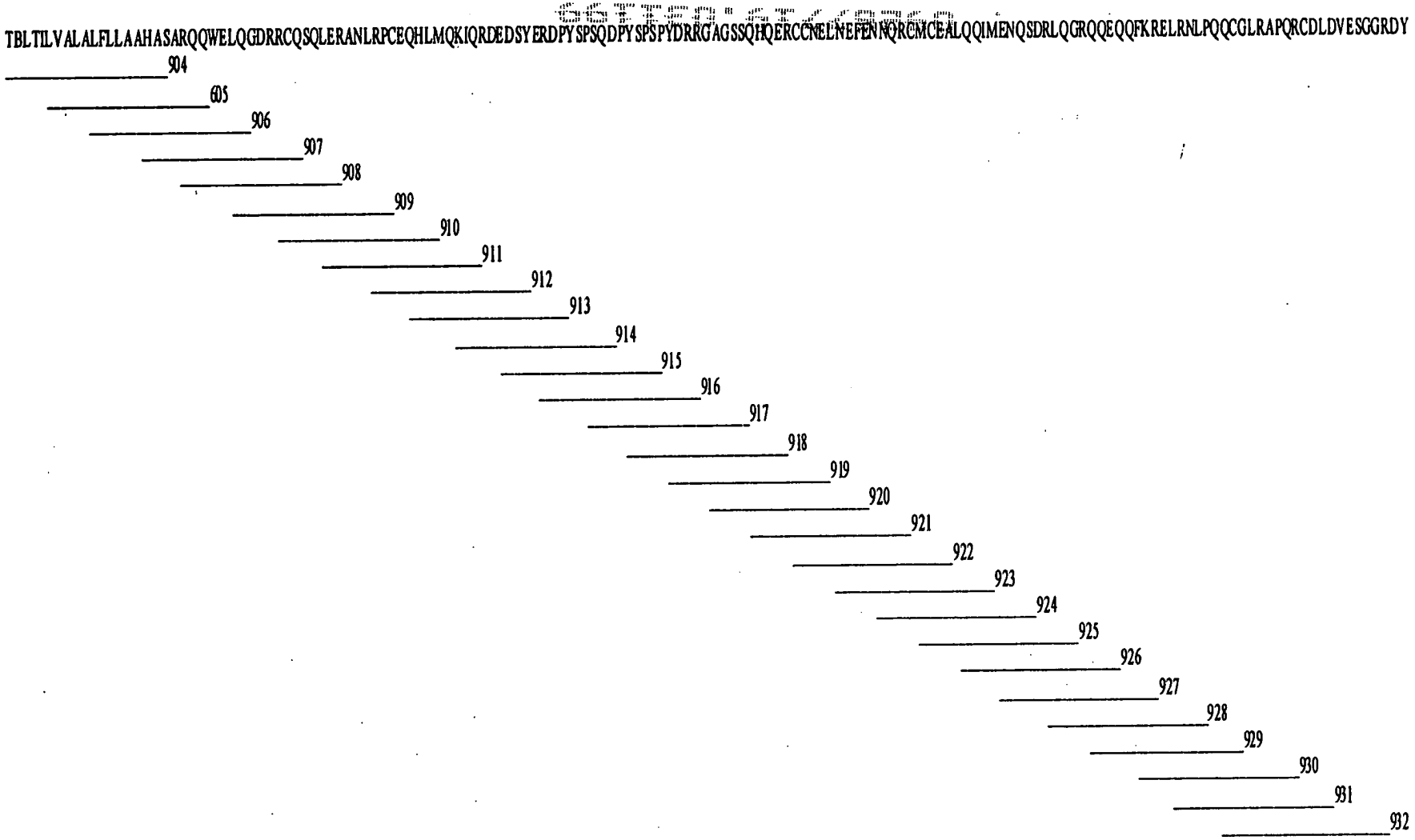


Fig.25 Synthetic overlapping peptides of Ara h 2. In order to determine the T-cell epitopes of peanut allergen Ara h 2, 29 different peptides representing the entire protein were synthesized. Each peptide was 20 amino acids long and was offset from the previous peptide by 5 amino acids. In this manner we were able to cover the entire protein sequence by overlapping peptides. The primary amino acid sequence of the Ara

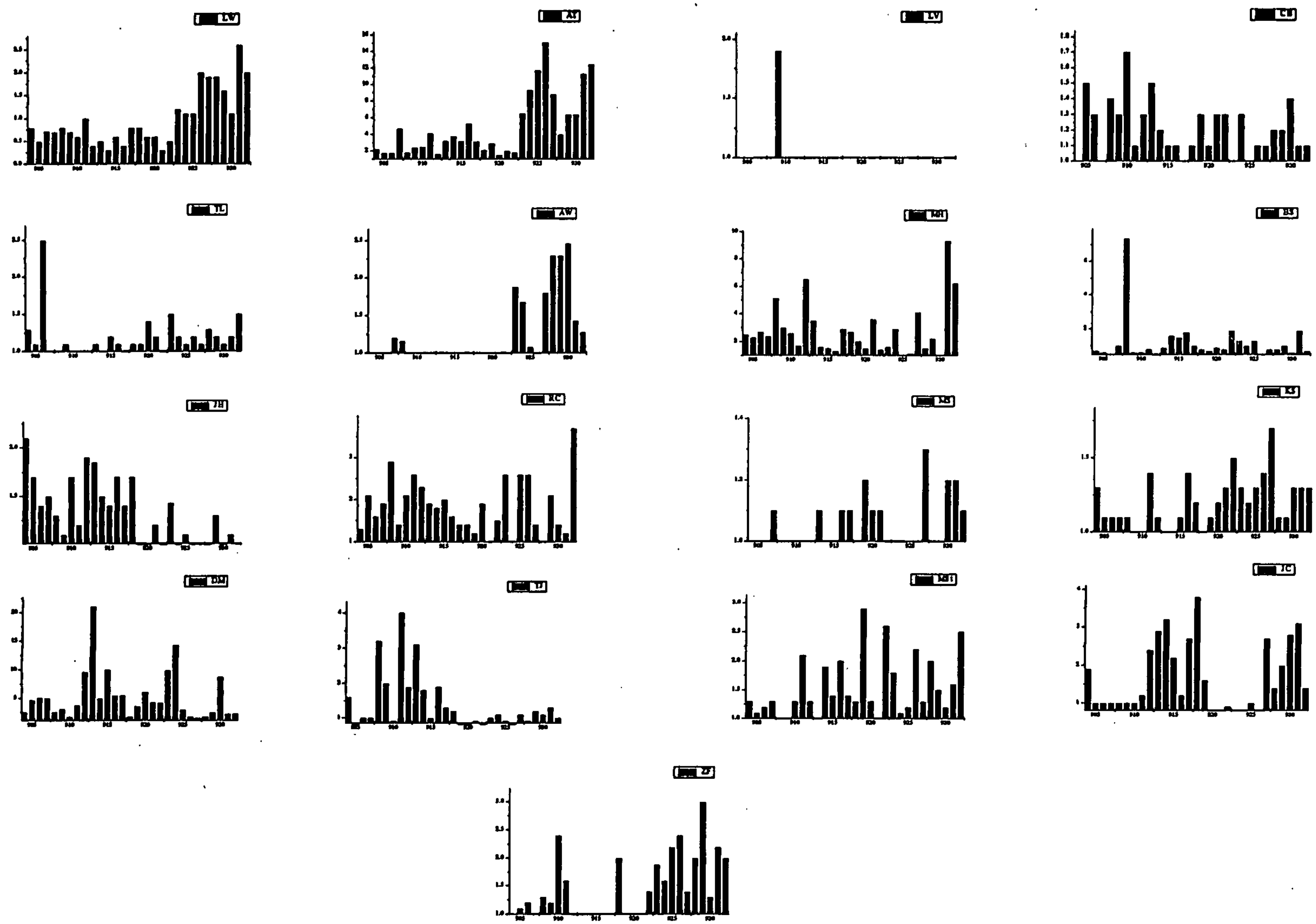
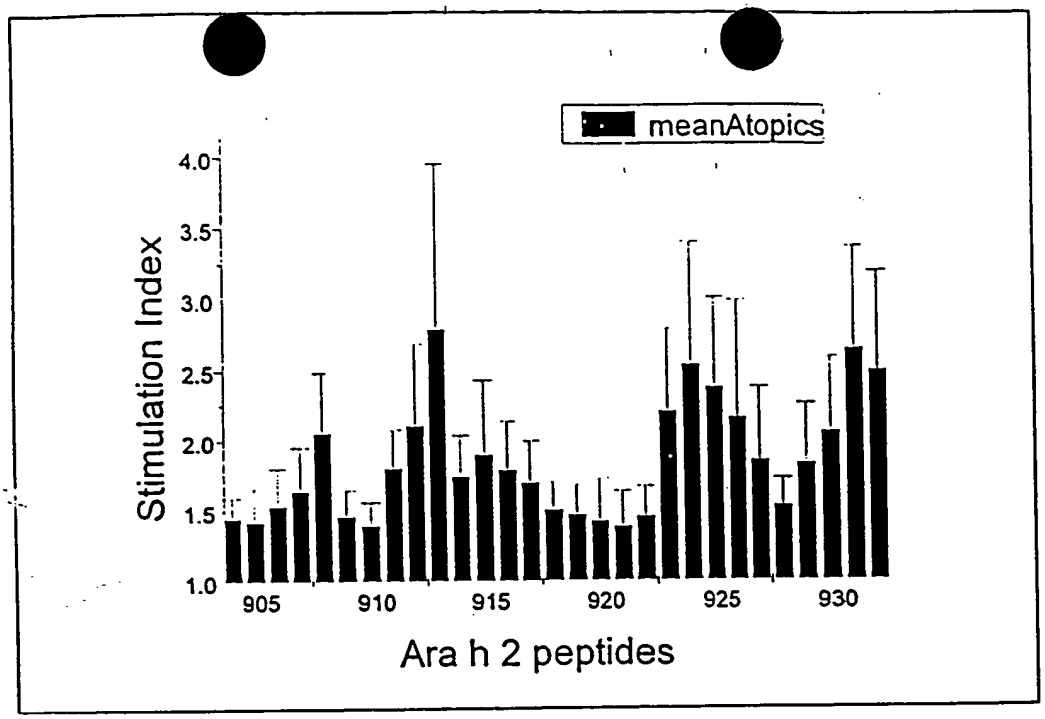
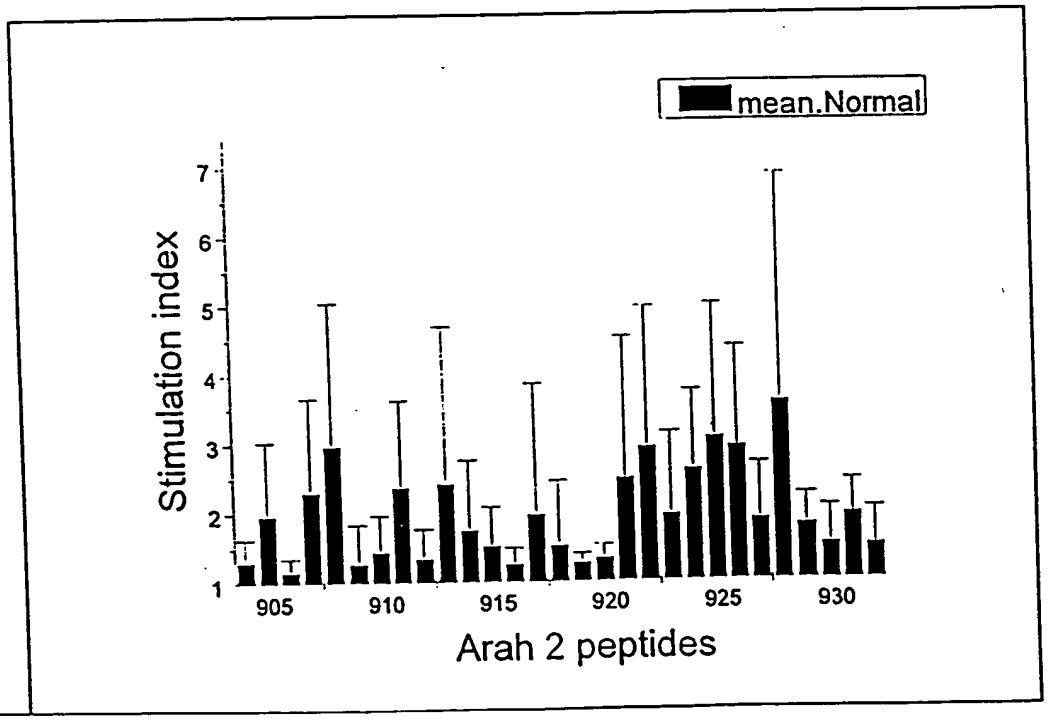


Fig. 26

Panel A



Panel B

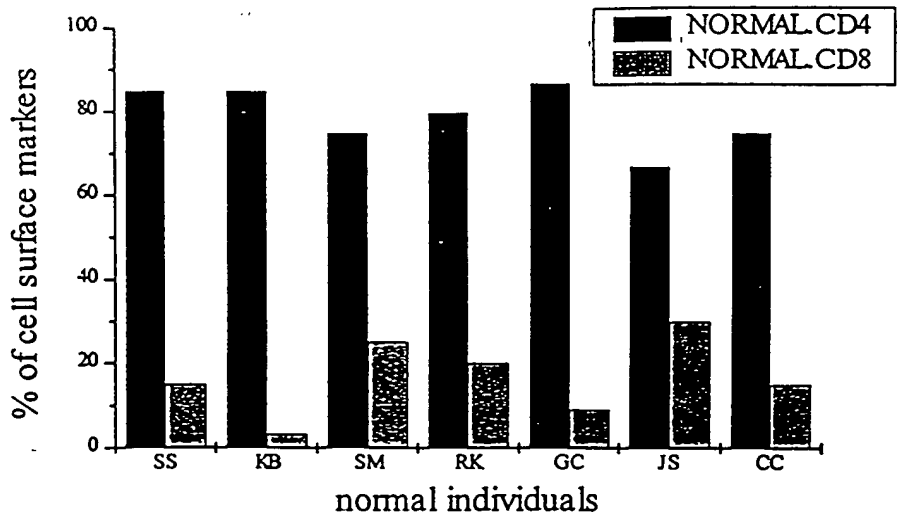


Identification of the Ara h 2 peptides that caused T-cell proliferation in the majority of patients tested.

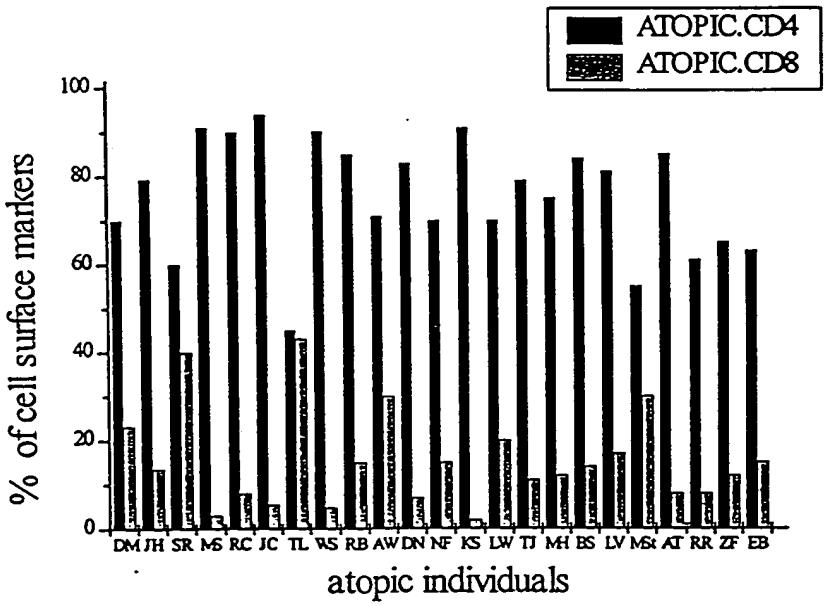
All of the data in Figure 2 was compiled and plotted as a stimulation index versus the Ara h 2 peptides. The mean proliferation and standard error of (panel A) 17 peanut allergic individuals and (panel B) 5 non-allergic individuals were calculated and plotted as mean stimulation index of atopic individuals versus the 29 overlapping peptides spanning the entire Ara h 2 protein from the amino-(peptide 904) to carboxyl terminus (peptide 932).

Fig. 27

Panel A



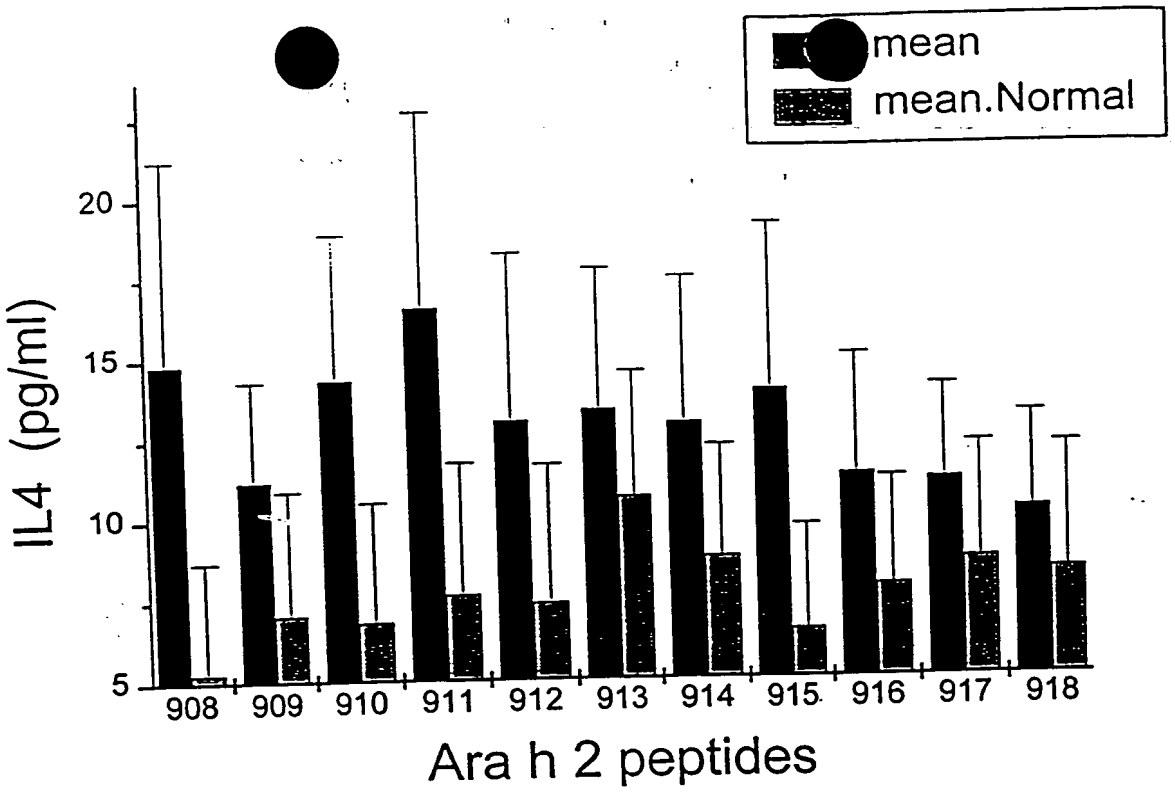
Panel B



The CD4$^+$ and CD8$^+$ profiles of the T-cell lines of peanut allergic individuals.

T cells were stained with FITC-labeled anti-CD4 and FITC-labeled anti-CD8 antibodies in order to determine the phenotype of the peanut specific T-cell lines established. FACS analysis was used to determine the percent of CD4$^+$ and CD8$^+$ cells in the peanut specific T-cell lines utilized in Ara h 2 epitope mapping and plotted versus the initials of the individual patients used to establish these cell lines. Panel A represents the CD4/CD8 profiles of T-cell lines established from allergic individuals while panel B represents the CD4/CD8 profiles of T-cell lines established from non-allergic individuals.

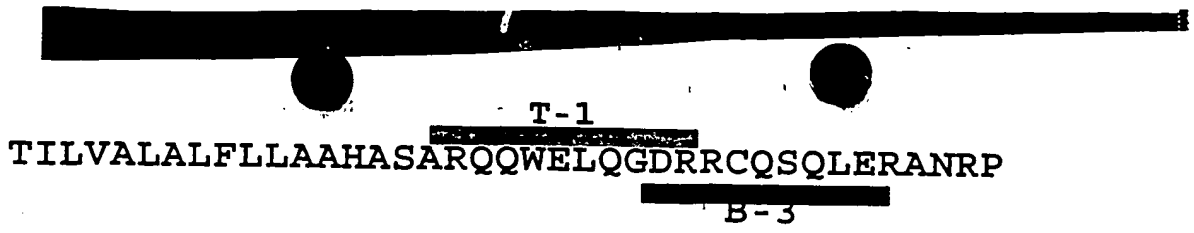
Fig. 28



The IL-4 secretion profiles of a representative sample of T cells.

The supernatant was collected from T-cells stimulated with immunodominant peptides and an ELISA assay was utilized to measure IL-4 concentrations in the media. IL-4 concentration is plotted versus the 29 overlapping peptides spanning the entire Ara h 2 protein from amino- (peptide 904) to carboxyl terminus (peptide 932).

Fig. 29



Comparison of the T-cell and B-cell epitopes of Ara h 2.

The primary amino acid sequence of the Ara h 2 protein is represented as the one letter amino acid code. The T-cell epitopes of Ara h 2 that have been identified in this study are depicted as bold, italicized letters and the immunodominant B-cell epitopes determined in previous work are underlined. In general, the IgE binding epitopes do not overlap with the T-cell epitopes.

Fig. 30

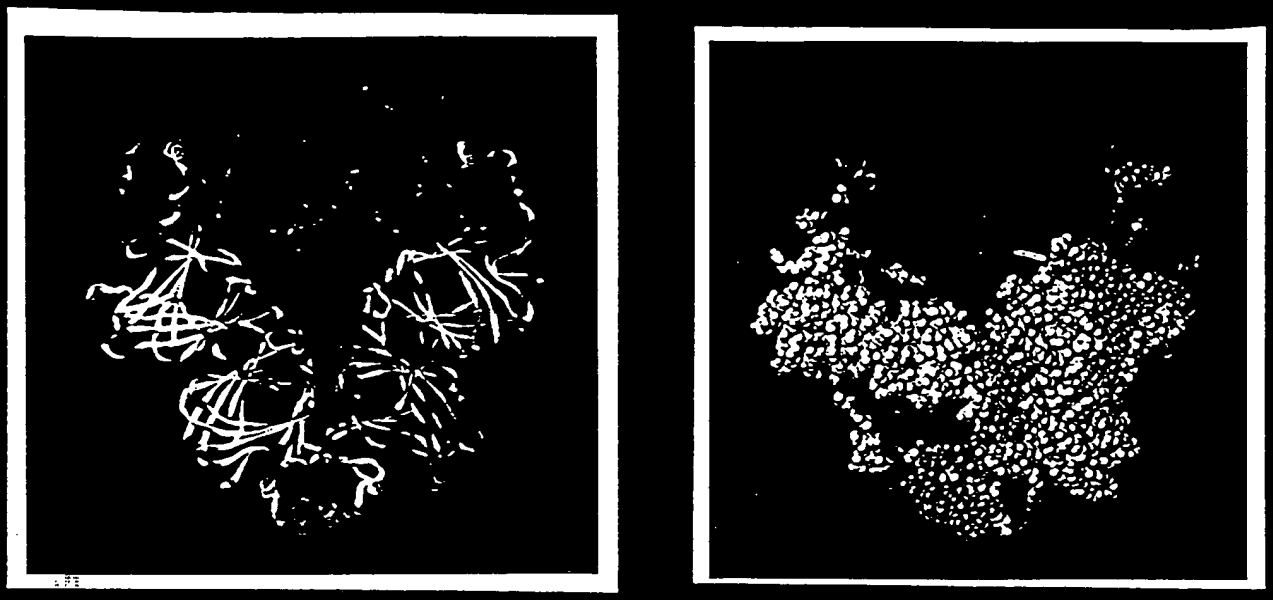


Fig. 31 Trimeric model of the Ara h 1 molecule.

It has been shown that other vicilin homologs form trimeric structures. Ara h 1 was also determined to form a trimeric structure by fluorescence anisotropy and cross linking experiments (see poster # 994). Ara h 1 was modeled in trimeric form by aligning the constructed model (see fig. 1 and 4) to monomers A, B and C of the trimeric template molecule phaseolin. The left panel is a ribbon diagram of the trimer with each monomer represented in a different color. The right panel is a space filled diagram where the epitope regions on the white monomer are shown in red and the epitope regions on the yellow monomer are shown in orange. This shows that a clustered epitope region on one side of a monomer joins the opposite clustered epitope region of another.

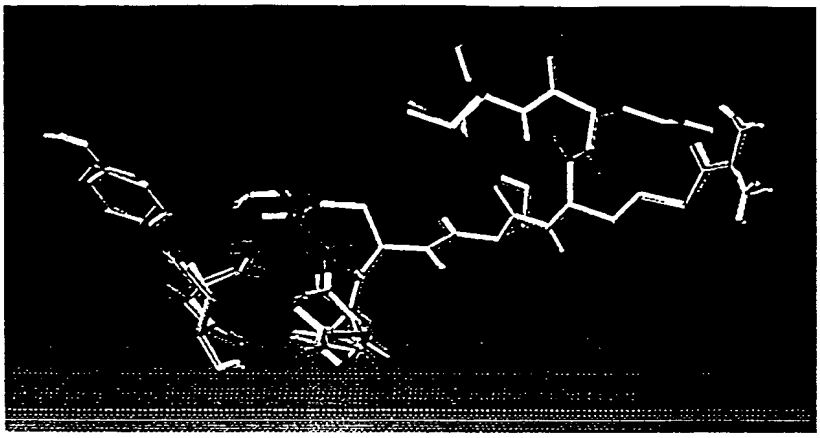


Fig. 32 Determination of residues targeted for mutagenesis in the Ara h 1 gene.

Residues that were found to abolish IgE binding through peptide mutagenesis (see Fig. 2 and Table 1) were mutated and analyzed in the molecular model to establish which residues would less likely interfere with protein stability. The wild type form of epitope 11, SYLQEFSRNT, is shown in white with the leucine chosen to be mutated in red. The gold peptide is the mutated form with a methionine substitution shown in purple. The mutant was locally annealed. This substitution is predicted to leave the molecule in the least disturbed conformation and has the lowest energy compared to the other residues that were shown to abolish IgE binding when mutated (shown in bold: SYLQEFSRNT). The other modeled epitopes were analyzed using the same method and one or more choices per epitope appeared to be suitable for mutagenesis without altering the tertiary structure of Ara h 1 significantly.

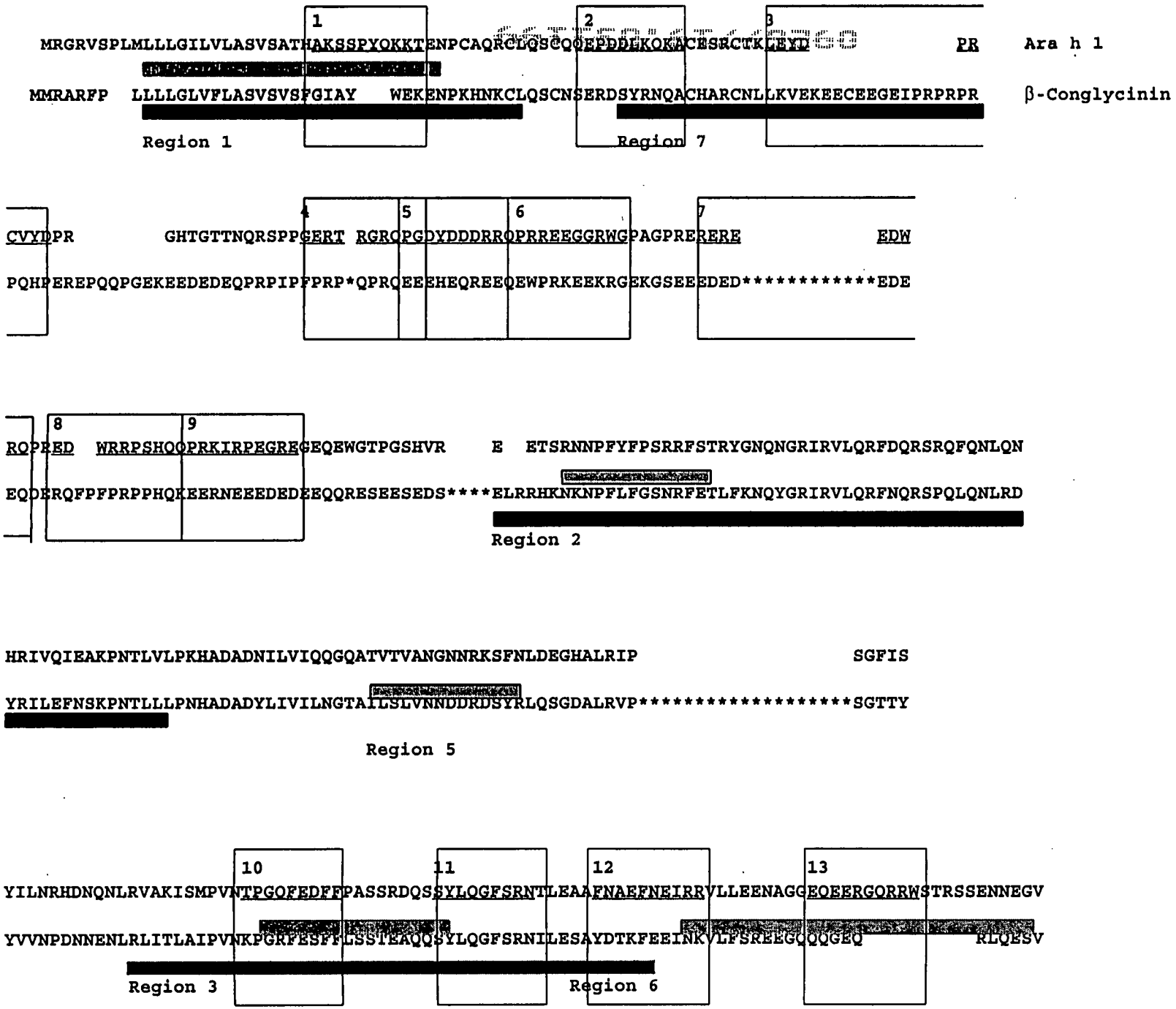
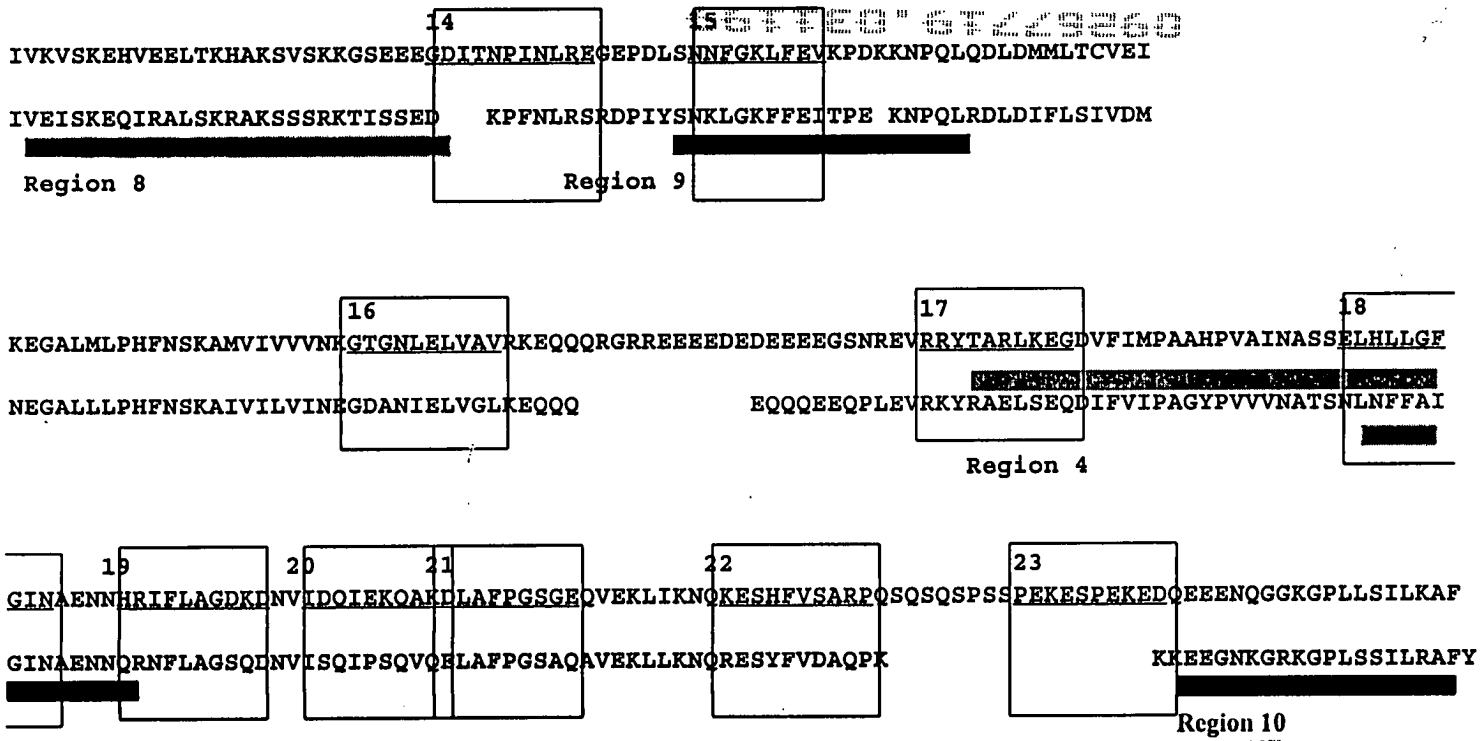

1
2
3
MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEPDDLKQKACESRCTKLEYD PR Ara h 1
MMRARFP LLLLGLVFLASVSVSPGIAY WEKENPKHNKCLQSCNSERDSYRNQACHARCNLLKVEKEECEEGEIPRPRPR β-Conglycinin
Region 1
Region 7
4
5
6
7
CVYDPR GHTGTTNQRSPPGERT RGRQPGDYDDDRRQPRREEGGRWGPAGPRERERE EDW
PQHPEREPQQPGEKEEDEDEQPRPIPFPRP*QPRQEEEHEQREEQEWPRKEEKRGEKGSEEEDED**********EDE
8
9
RQPRED WRRPSHQQPRKIRPEGRE GEQEWGTPGSHVR E ETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFDQRSRQFQNLQN
EQDERQFPFPRPPHQKEERNEEEDEDEEQQRESEESEDS****ELRRHKNKNPFLFGSNRFETLFKNQYGRIRVLQRFNQRSPQLQNLRD
Region 2
HRIVQIEAKPNTLVLPKHADADNILVIQQGQATVTVANGNNRKSFNLDEGHALRIP SGFIS
YRILEFNSKPNTLLLPNHADADYLIVILNGTATLSLVNNDDRDSYRLQSGDALRVP**********SGTTY
Region 5
10
11
12
13
YILNRHDNQNLRVAKISMPVNTPGQFEDFFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWSTRSSENNEGV
YVVNPDNNENLRLITLAIPVNKPGRFESFFLSSTEAQQSYLQGFSRNILESAYDTKFEEINKVLFSREEGQQQGEQ RLQESV
Region 3
Region 6

Fig. 33A



SOYBEAN SEQUENCE HOMOLOGY TO ARA H 1
SOYBEAN AND PEANUT POSITIVE REGIONS
15-MERS X 8 SPOTS ANALYSIS

Soybean IgE Positive Binding Regions

Peanut IgE Positive Binding Regions

Ara h 1 Epitopes

Fig. 33B

SEQUENCE HOMOLOGY OF ARA H I IgE EPITOPES IN REGIONS OF BETA CONGLYCININ

EPITOPE 1	EPITOPE 2	EPITOPE 3	EPITOPE 4	EPITOPE 5
AKSSPYQKKT	QEPDDLKQKA	LEYD PRLVYD	GERTRGRQPG	PSDYDDDRRQ
GIAY WEK	SERDSYRNQA	LKVEKEECEEGEIPRPRPRPQHP	FPRPQPRQEE	EEEHEQREEQ
	* * *	* **	* **	*

EPITOPE 6	EPITOPE 7	EPITOPE 8	EPITOPE 9	EPITOPE 10
PRREEGGRWG	RERREEDWRQP	EDW RRPSHQQ	QPRKIRPEGR	TPGQFEDFFP
EWPRKEEKRG	EDEDEDEDEQ	RQFPFPRPPHQK	KEERNEEEDE	KPGRFESFFL
*	*	** **	*	** ** **

EPITOPE 11	EPITOPE 12	EPITOPE 13	EPITIPE 14	EPITOPE 15
SYLQEFSRNT	FNAEFNEIRR	EQEERGQRRW	DITNPINLRE	NNFGKLFEVK
SYLQGFSRNI	YDTKEEINKV	QQGEQRILQE	KPFNLRS	NKLGKFFEIT
**** ****		* *	* ***	* ** **

EPITOPE 16	EPITOPE 17	EPITOPE 18	EPITOPE 19	EPITOPE 20
GTGNLELVAV	RRYTARLKEG	ELHLLGFGIN	JRIFLAGDKD	IDQIEKQAKD
GDANIELVGL	RKYREELSEQ	NLNFFAIGIN	QRNFLAGSQD	ISQIPSQVQE
* * ***	* * *	* ***	**** *	* ** *

EPITOPE 21	EPITOPE 22	EPITOPE 23
KDLAFPGSGE	KESKFVSARP	PEKESPEKED
QELAFPGSAQ	RESYFVDAQP	K
******	** ** * *	

Fig. 34

cDNA CLONING

Soybean seeds, Glycinus max, Hutchinson variety, were obtained from a local health food store, frozen in liquid nitrogen, ground to a fine powder, and the RNA extracted using the method of Nedergaard et al (Mol Immunol 29:703,1992). Briefly, 2 g frozen seed powder was added to 10 mls buffer (250 mM sucrose, 200 mM Tris-HCl, pH 8.0, 200 mM KCl, 30 mM $MgCl_2$, 2% polyvinylpyrrokidone-40 and 5 mM 2-mercaptoethanol) and equilibrated with 10 ml fresh phenol (4°C). The suspension was homogenized and 10 ml of chloroform added with shaking for 5 min at RT. Phases were separated by centrifugation, 10k g for 20 min at 4°C and the aqueous phase transferred to a clean test tube and extracted 2x with equal volumes of chloroform/phenol. Nucleic acids were precipitated with sodium acetate/ethanol at -20°C overnight. The precipitates were collected by centrifugation at 13k g for 20 min at 4°C, washed with 70% ethanol and dried. Samples run in parallel were pooled in water and made 3M in LiCl, and the RNA precipitated for 4 hr at -20°C. The precipitate was collected by centrifugation outlined above and resuspended in distilled water. Fifty microliters of the RNA suspension was withdrawn for OD260/280 measurements and the RNA analyzed by agarose gel electrophoresis. Three aliquots representing a total of approximately 3.0 mgs total RNA was sent to STRATAGENE for purification of mRNA and the preparation of a Uni-Zap XR custom library.

The expression custom library was screened with serum from soybean-sensitive individuals and positive clones subcloned to homogeneity with respect to IgE-binding. Five clones were isolated from an initial screen and the plasmids purified from LB/ampicilin broth cultures using an Ameresco kit. The plasmid DNA from each clone was PCR amplified and analyzed in agarose gels. Two plasmid preparations had relative bp of approximately 1400 and the remaining three 1500 bp.

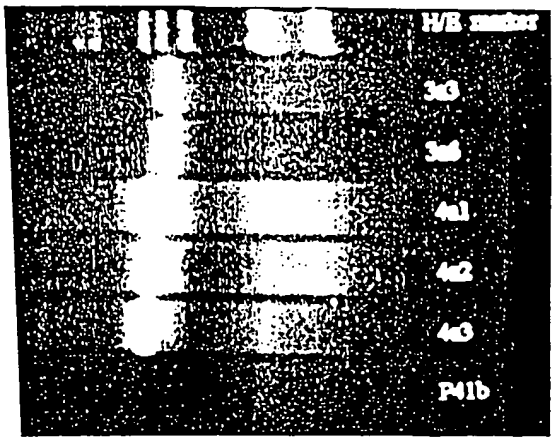


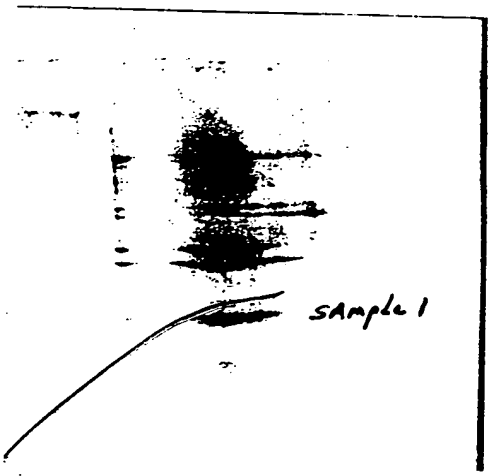
Fig. 35

PCR AMPLIFIED PLASMIDS ISOLATED FROM SOYBEAN CDNA EXPRESSION LIBRARY

ALLE[redacted]EN IDENTIFICATION BY 2[redacted]SDS-PAGE

A crude soybean extract was applied to a 12.5% preparative SDS-PAGE gel and electrophoresed using a BIO-RAD prep cell. Five ml fractions were collected and aliquots were electrophoresed into a Pharmacia 24-well 10% horizontal gel, electrophoretically transferred to a nitrocellulose membrane, the remaining sites blocked using PBS/0.05% Tween 20, and analyzed for IgE-binding using serum from soybean-sensitive individuals. Fractions that bound IgE were dialyzed against 100mM ammonium bicarbonate (x4 x 4 liters) for 24 hours, lyophilized, reconstituted in distilled water and analyzed by 2-D (isoelectric focusing in the first dimension, pH 3-7, followed by a 4-20% SDS-PAGE gel molecular weight separation in the second) in duplicate. The proteins in the duplicate gels were transferred to nitrocellulose membranes, one was stained with Coomassie blue for protein identification and the other was prepared for IgE immunoblot analysis. IgE-binding proteins were identified by radiolabeled anti-IgE and X-ray autoradiography. Positive IgE-binding proteins by autoradiography were compared to the Coomassie stained gel protein profile. The stained blot was submitted to the Yale Biotechnology Center for amino acid sequencing. The results of this analysis revealed a 20-22kD protein with significant homology to the A2B1a glycinin protein family. Additional samples are being assessed for activity and identification.

A: Coomassie blue stained 2-D SDS-PAGE gel



B: IgE immunoblot of 2-D SDS-PAGE blot

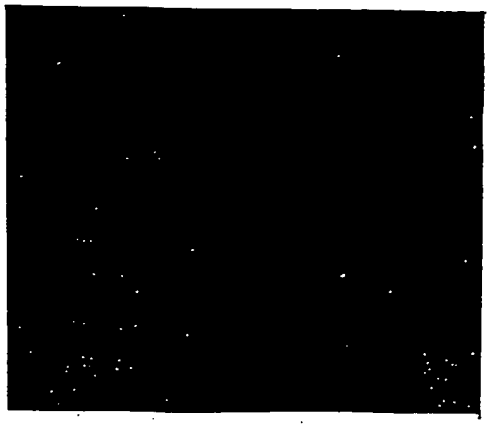
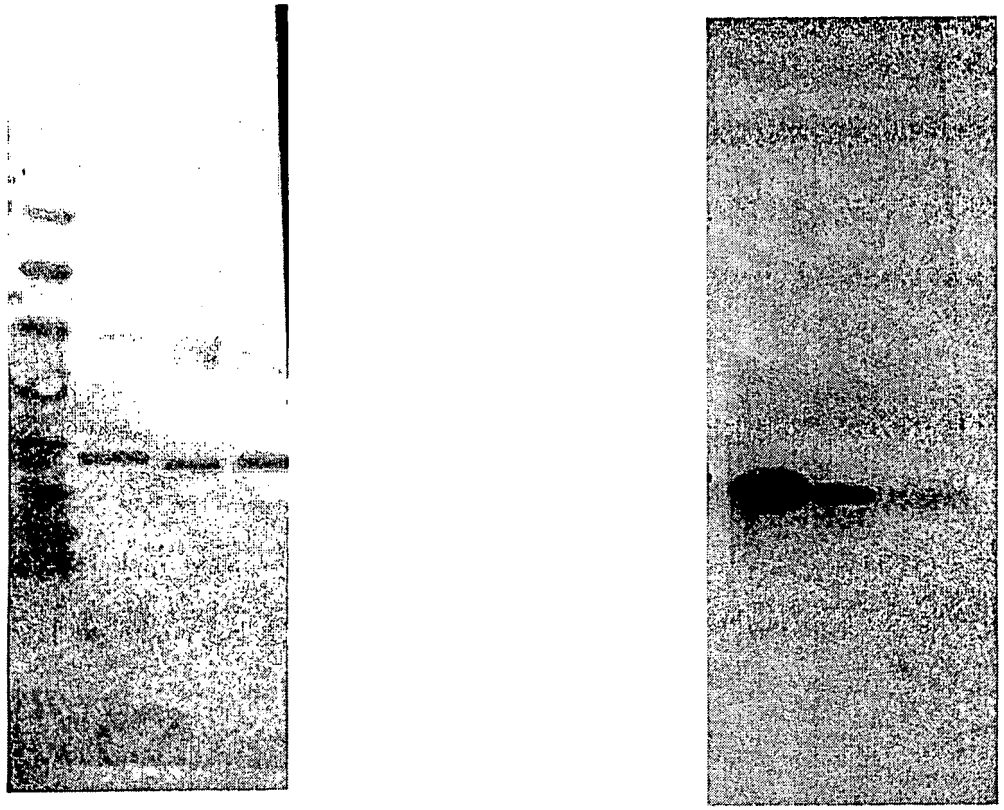


Fig. 36

Figure 37A

IgE BINDING OF rAra h 2 PROTEINS IN WESTERN BLOT ANALYSIS

T7 tag **Serum IgE**



MW 10 6 0 **10 6 0**

Number of epitopes

Figure 37 B

SERUM IgE BINDING OF rAra h 2 PROTEINS IN INDIVIDUAL PATIENTS

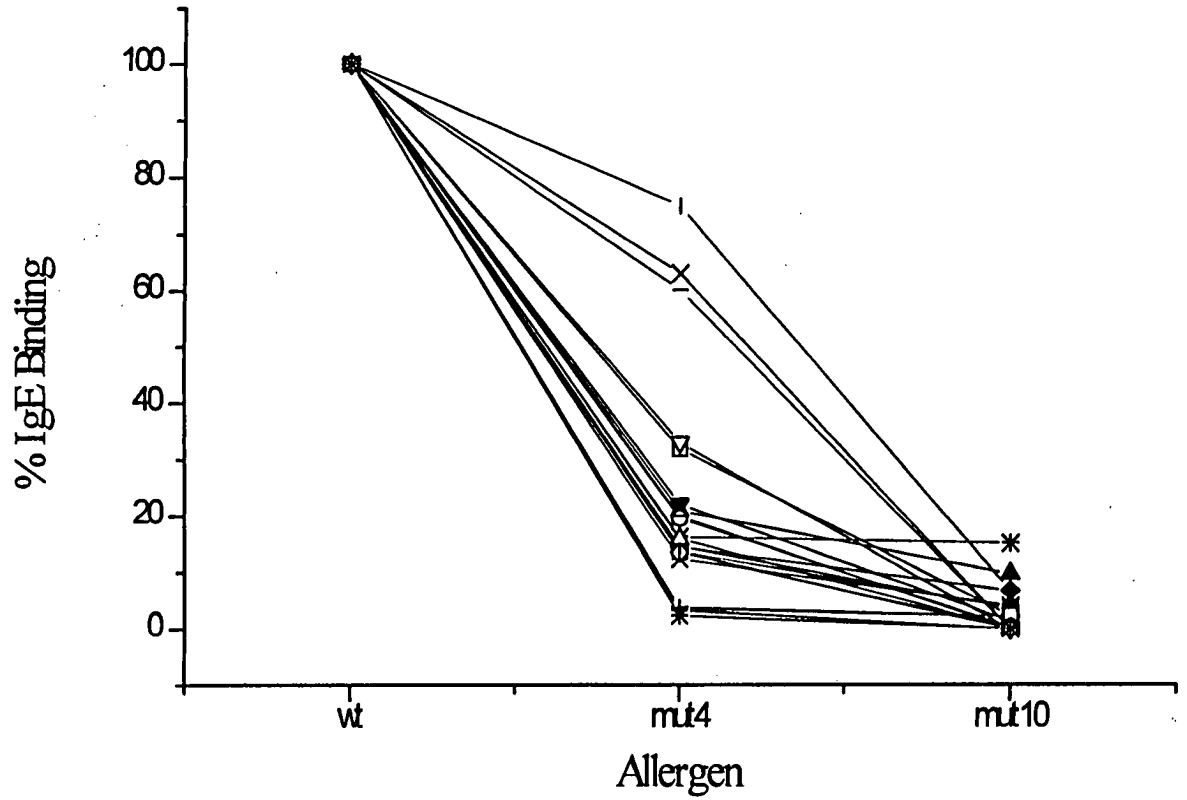


Fig. 38 Upper Panel

Fig. 38 Lower Panel

Fig 37 Upper Panel

Ara h 1- Pepsin/Chymotrypsin Sites

MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEP
DDLKQKACEESRCTKLEYDPRCVYDPRGHTGTTNQRSPPGERTRGRQPGD
YDDDRRQPRREEGGRWGPAGPRERERREEDWRQPREDWRRPSHQQPRKIRP
EGREGEQEWGTPGSHVREETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFD
QRSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQGQATVTVANGN
NRKSFNLDEGHALRIPSGFISYILNRHDNQNLRAAKISMPVNTPGQFEDFFPA
SSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWST
RSSENNEGVIVKVSKEHVEELTKHAKSVSKKGSEEEGDITNPINLREGEPDLS
NNFGKLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVV
NKGTGNLELVAVRKEQQQRGRREEEEDEDEEEEGSNREVRRYTARLKEGD
VFIMPAAHPVAINASSELHLLGFGINAENNHRIFLAGDKDNVIDQIEKQAKD
LAFPGSGEQVEKLIKNQKESHFVSARPQSQSQSPSSPEKESPEKEDQEEENQG
GKGPLLSILKAFN

Fig. 39 Lower Panel

Ara h 1- Trypsin Sites

MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEP
DDLKQKACEESRCTKLEYDPRCVYDPRGHTGTTNQRSPPGERTRGRQPGD
YDDDRRQPRREEGGRWGPAGPRERERREEDWRQPREDWRRPSHQQPRKIRP
EGREGEQEWGTPGSHVREETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFD
QRSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQGQATVTVANGN
NRKSFNLDEGHALRIPSGFISYILNRHDNQNLRAAKISMPVNTPGQFEDFFPA
SSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWST
RSSENNEGVIVKVSKEHVEELTKHAKSVSKKGSEEEGDITNPINLREGEPDLS
NNFGKLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVV
NKGTGNLELVAVRKEQQQRGRREEEEDEDEEEEGSNREVRRYTARLKEGD
VFIMPAAHPVAINASSELHLLGFGINAENNHRIFLAGDKDNVIDQIEKQAKD
LAFPGSGEQVEKLIKNQKESHFVSARPQSQSQSPSSPEKESPEKEDQEEENQG
GKGPLLSILKAFN

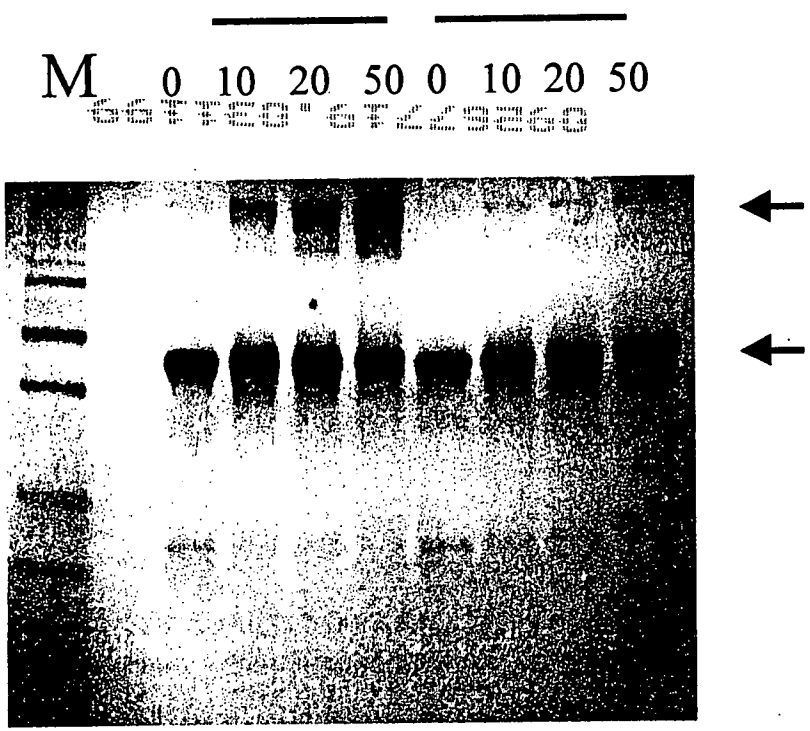


Figure 40 The Ara h 1 trimer is unstable at pH2.

In order to assess the stability of the Ara h 1 trimer at pHs that would be encountered in the human stomach, cross-linking experiments were performed using purified Ara h 1 protein suspended in a pH 2.1 buffer. Purified Ara h 1 (2 mM) was suspended in 500 µl of either a pH 2.1 buffer or a pH 7.6 buffer and allowed to incubate for one hour at room temperature. Cross-linking was performed using 5% DSP in DMF for varying lengths of time (10, 20, or 50 seconds). Results indicate that the Ara h 1 trimer is unstable at acidic pHs that would be encountered in the human stomach but that the monomer is stable at this pH. Further experiments indicate that the monomer is stable at pH 2.1 for greater than 8 hours at 37°C.

Figure 4A

EXPRESSION OF RECOMBINANT Ara h 2 PROTEIN IN *E. Coli*

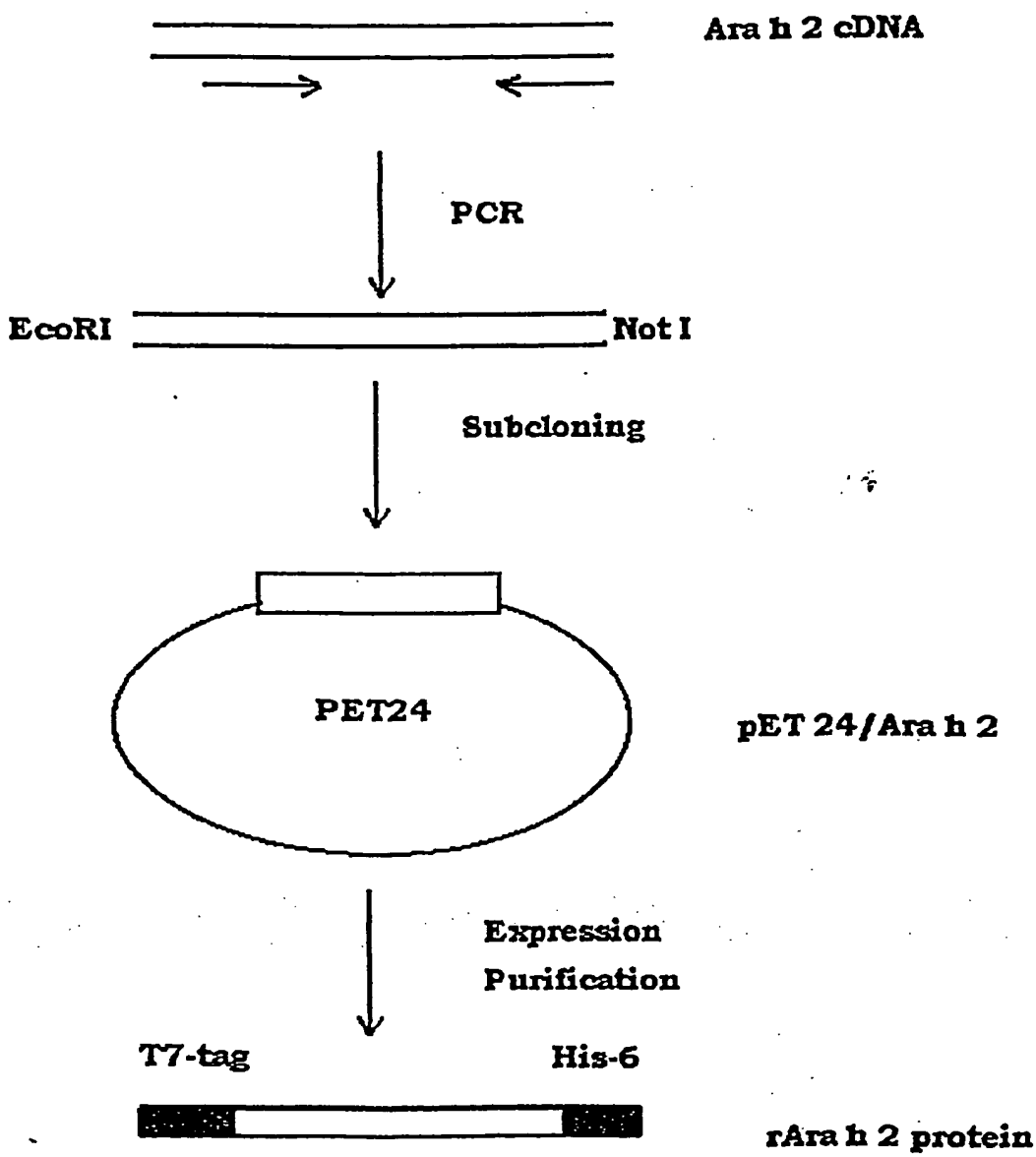
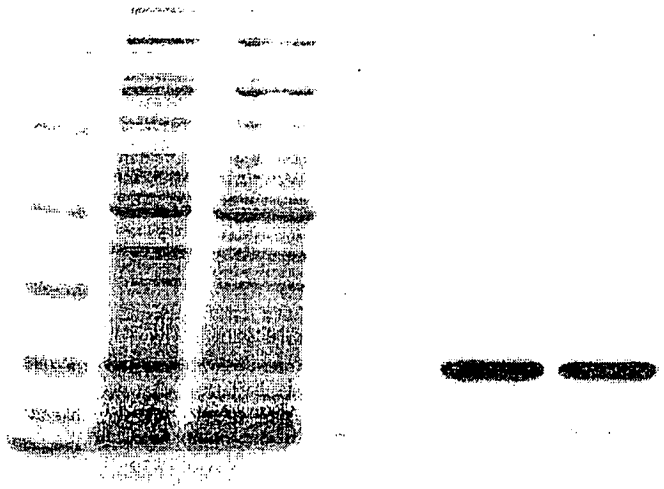


Figure 4/ B

PURIFICATION OF RECOMBINANT Ara h 2 PROTEINS ON A Ni-COLUMN



MW 1 2 3 4 5 6

Figure 42
INHIBITION OF IgE BINDING TO NATIVE Ara h 2 PROTEIN

0.5 ug of the native Ara h 2 protein purified from crude peanut extracts were loaded onto nitrocellulose membrane using a slot-blot apparatus. Membranes were incubated with patient serum pool (1:20) in the presence or absence of different concentrations of wild type or mutated recombinant Ara h 2 proteins. Membranes were probed for the bound IgE with ^{125}I anti-human IgE antibody. Laser densitometry of the autoradiograms was used to quantitate the relative amounts of IgE binding.

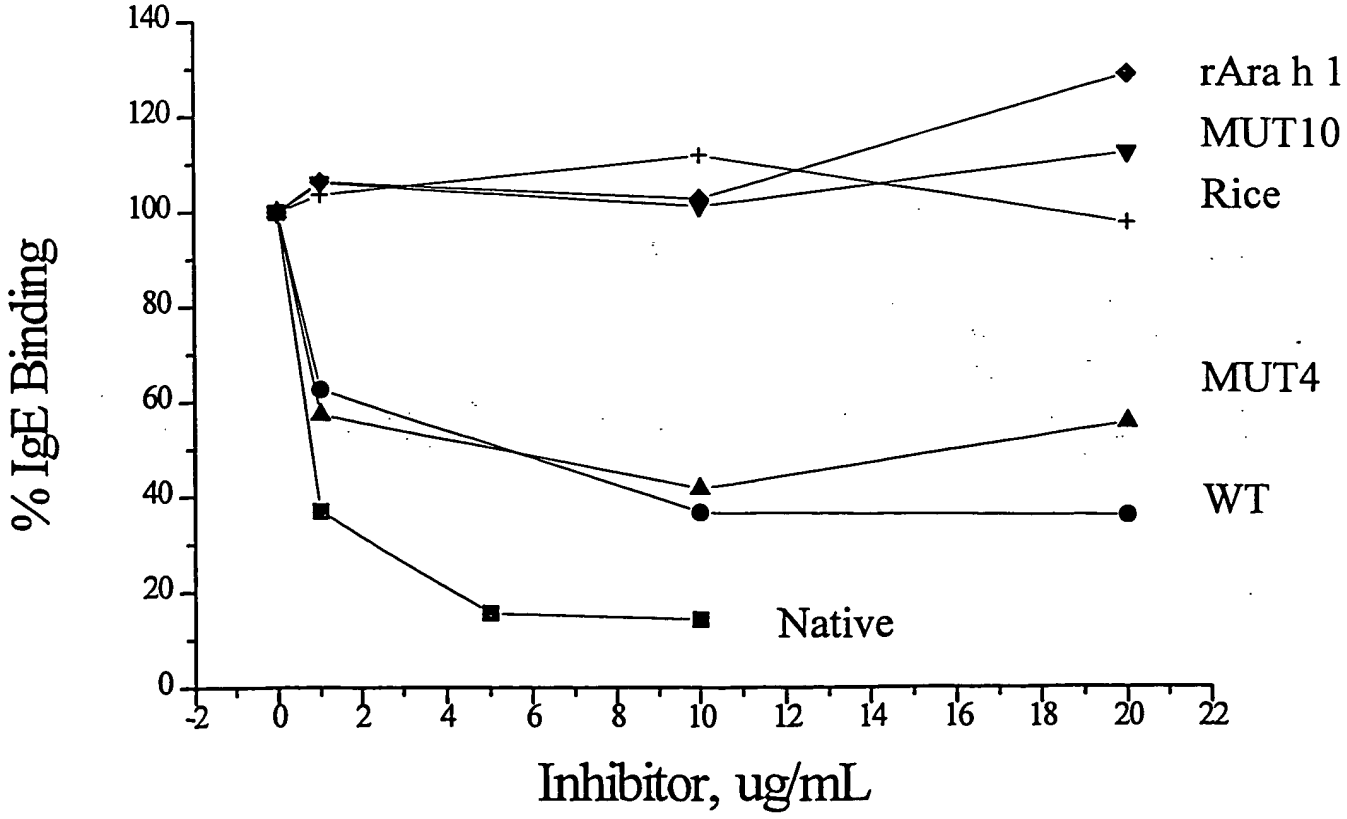
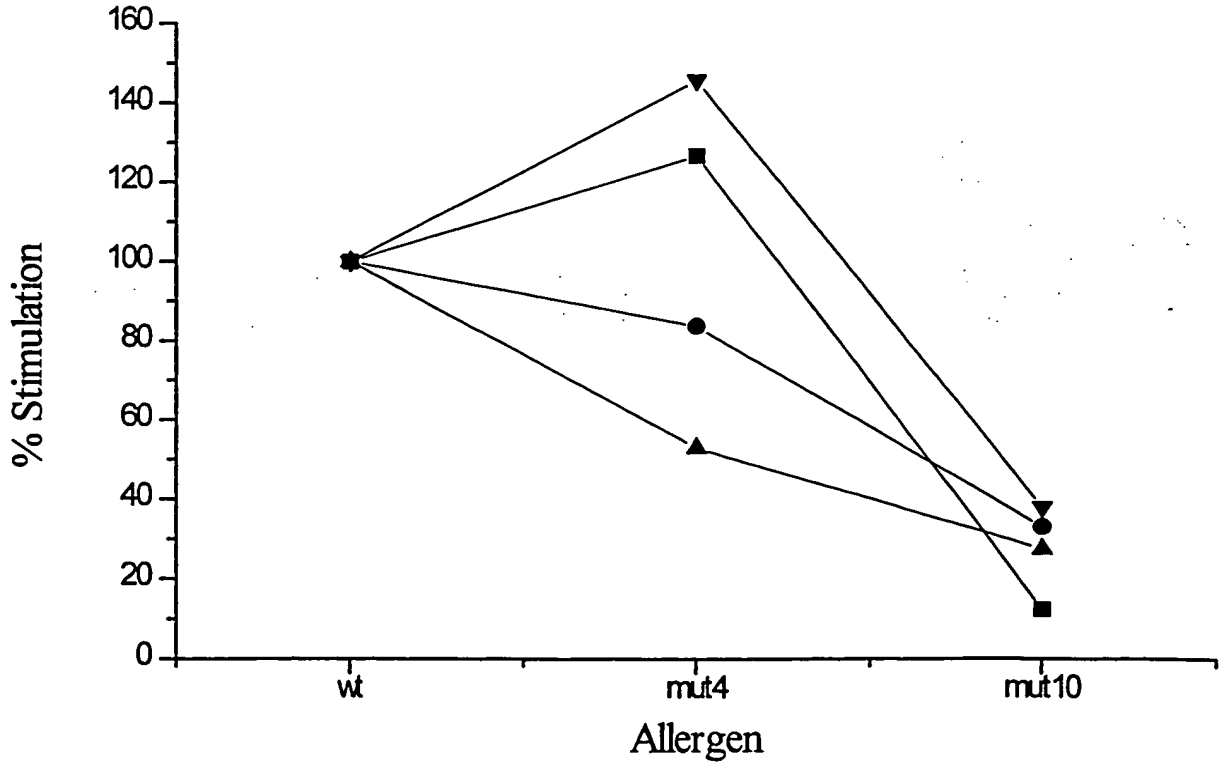
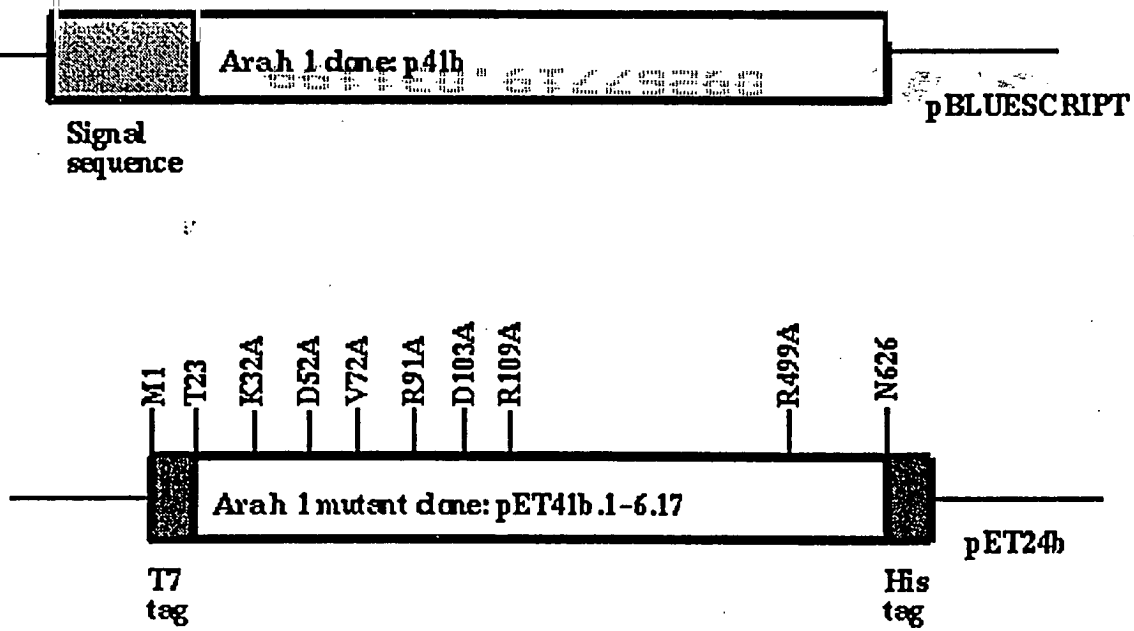


Figure 43
PROLIFERATION OF PBMCs FROM PEANUT SENSITIVE PATIENTS

PBMCs were isolated from heparinized venous blood of peanut-sensitive patients by density gradient centrifugation on Ficoll. $2x10^5$ cells per well were incubated in triplicates for 7 days in RPMI media with 5% human AB serum in the presence of 10 ug/ml of the native Ara h 2 protein purified from the crude peanut extract or recombinant Ara h 2 proteins purified from *E.coli.* Cells incubated in media only were used as a control. Proliferation was measured by the incorporation of tritiated thymidine. Stimulation index is calculated as a ratio of radioactivity for the cells growing in the presence of allergen to that for the cells growing in media alone.





Epitope	Mutation
1	K32A
2	D52A
3	V72A
4	R91A
5	D103A
6	R109A
17	R499A

Figure 11 Wild type and mutant Ara h 1 plasmid constructs.

The top diagram represents the original Ara h 1 p41b construct. It consists of the Ara h 1 cDNA insert within the PBLUESRIPT SK- vector. Noted are the Met1, Thr23, and Asn626 positions. The residues Met1-Thr23 represent the Ara h 1 signal sequence and Asn626 is the C-terminal residue. The lower diagram represents the mutant Ara h 1 construct pET41b.1-6.17. It was constructed by inserting a PCR product into the pET24b expression vector. Noted are the epitope mutation positions (not drawn to scale) and the positions of the T7 and His tags. The particular mutations within each epitope are listed under the diagrams.

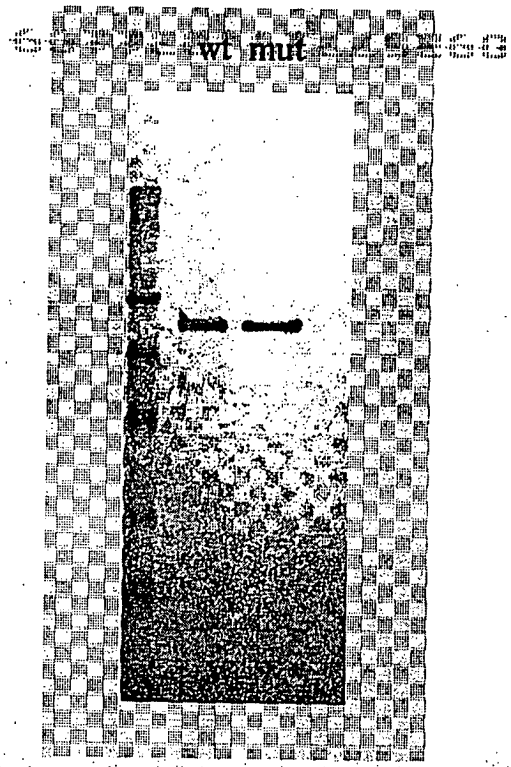
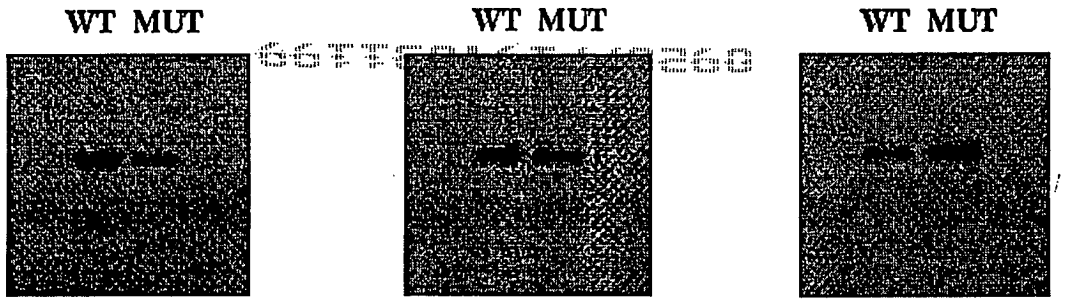


Figure 45 Wild type and mutant protein western blot control.

A western blot control was performed on the wild type and mutantAra h 1 recombinant proteins to ensure that an equal amount of each protein was used in these studies. Novex see-blue molecular weight markers were visualized by Coomassie staining in the first lane. Equal amounts of wild type (middle lane) and mutant (right lane) were detected by probing with anti-T7 antibody alkalinephosphatase conjugate. Both proteins migrate at their expected molecular weights (65 kDa).



Patient 1

Mutant epitopes:
1, 4, 5, 17

WT epitopes:
8, 13

Patient 2

Mutant epitopes:
2, 3, 4, 17

WT epitopes:
14, 18

Patient 3

Mutant epitopes:
4, 5, 17

WT epitopes:
11, 14, 18, 19, 20 ,22

Figure 16 Mutation of the Ara h 1 protein leads to altered IgE binding.

Three western blots of wild type (left lane) and mutant (right lane) recombinant proteins probed with individual peanut-sensitive patient sera are shown. The epitopes that each patient recognized are indicated below each blot. Mutant epitopes corresponds to the epitopes that the patient recognized that were altered in the mutant protein. WT epitopes corresponds to epitopes that were recognized by the patient, but were not changed in the mutant protein. In the first panel IgE binding was decreased. In the second panel binding was roughly equal. In the third panel binding was increased.

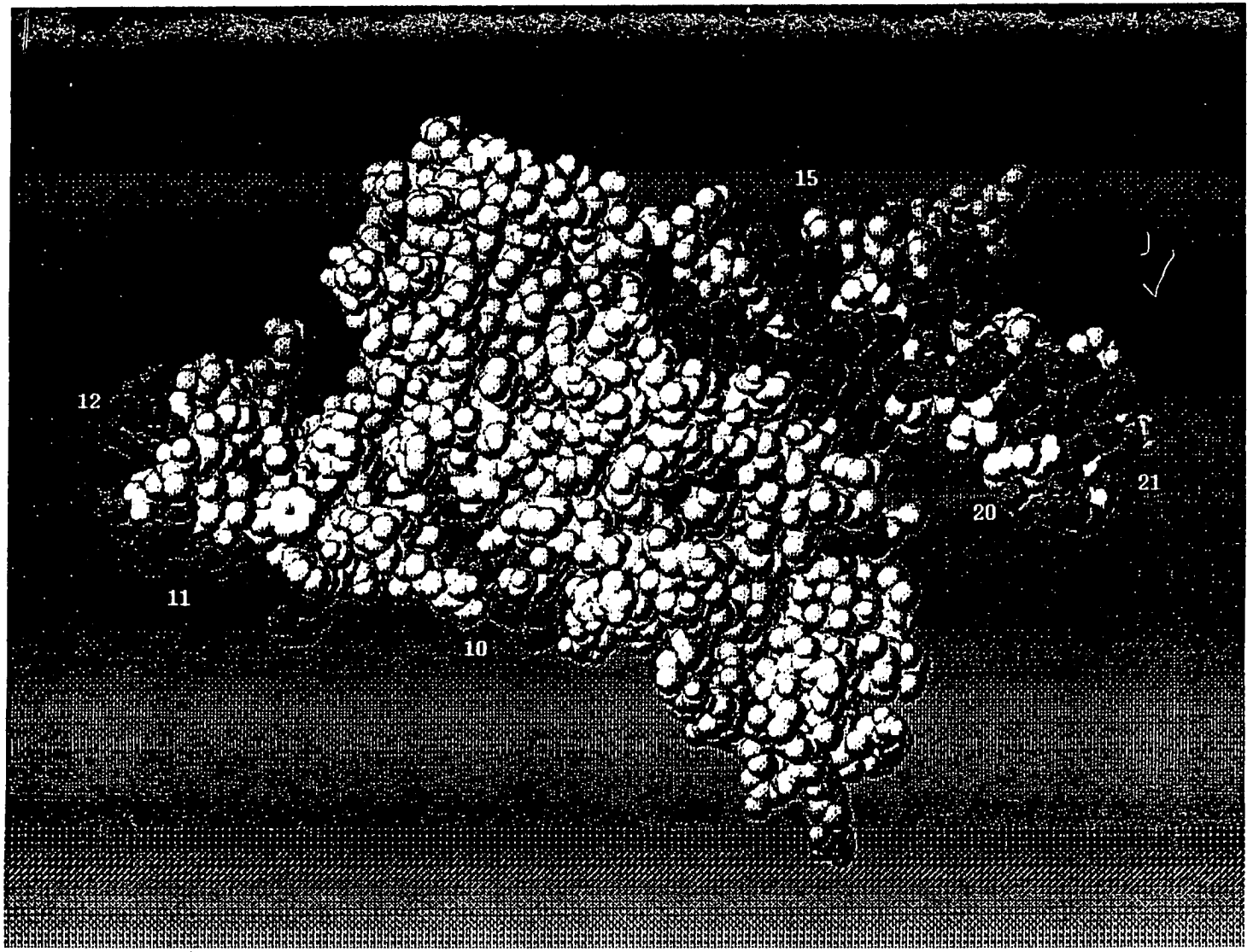


Figure 47 Tertiary structure model of the Ara h 1 protein.

A space-filled model of the middle and C-terminal domains of the Ara h 1 allergen is shown. The red areas represent the IgE binding epitopes. The yellow atoms represent residues that were determined to be critical for IgE binding to occur. The numbers correspond to some of the epitopes listed in Table 1.

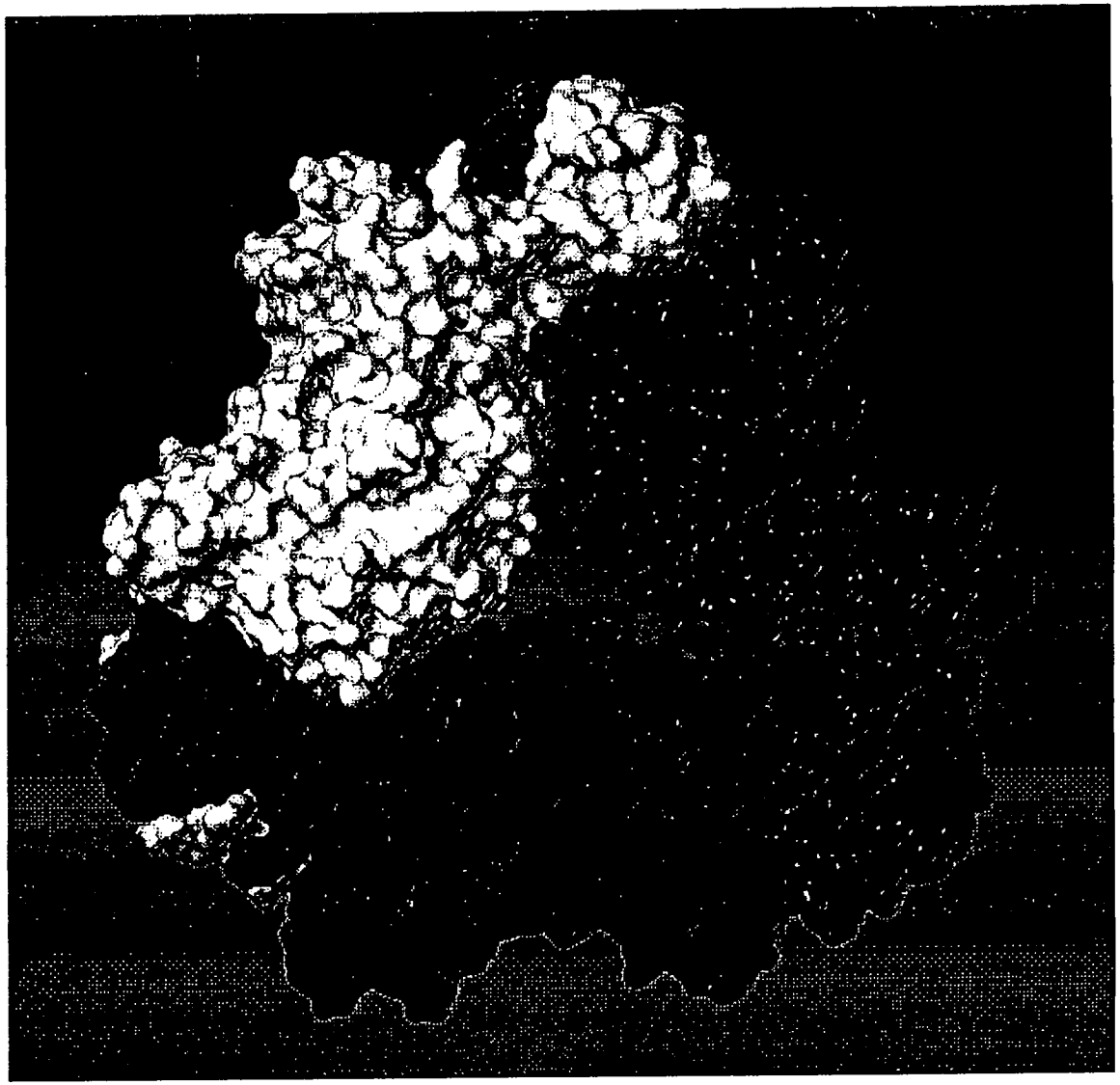


Figure 48 Contoured surface diagram of the trimeric Ara h 1 model.

A contoured surface of each Ara h 1 monomer is shown in a different color. The trimeric structure is based on the phaseolin trimer structure. The alpha helical bundles and adjacent beta sheets form the interface of monomer-monomer contact.

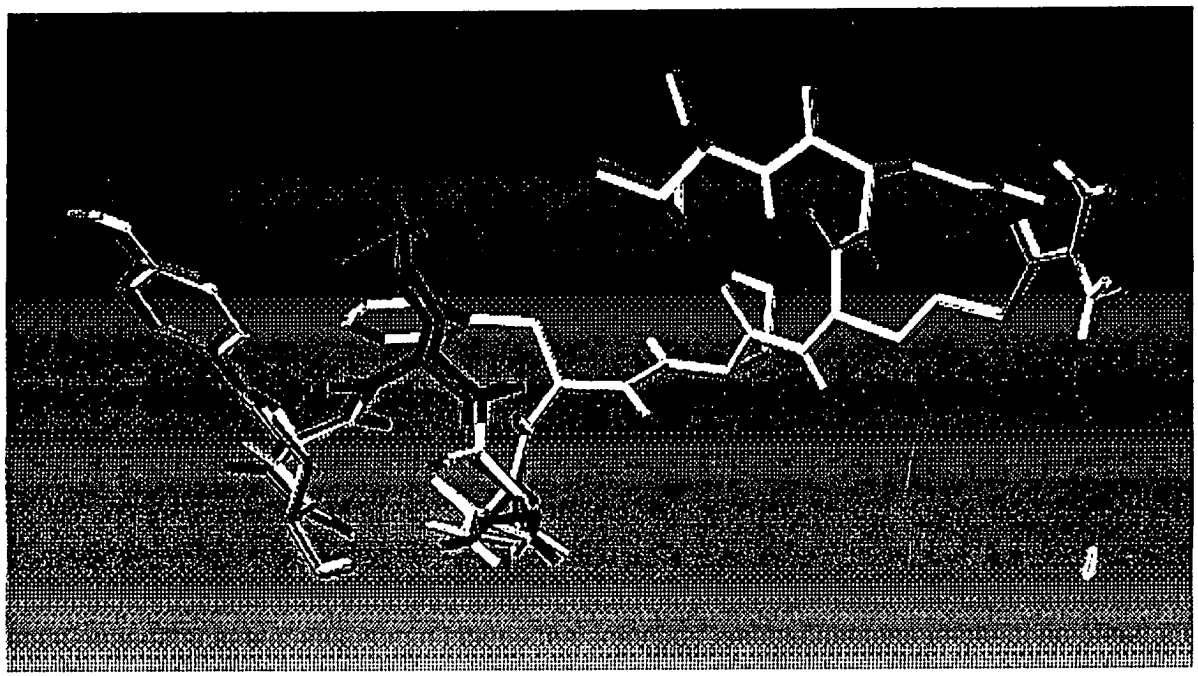


Figure 49 Calculated effect of L313M mutation on epitope 11.

A stick diagram of epitope 11 (SYLQGFSRNT) is shown in yellow with the exception ofLeu 313 which is shown in red. A mutant, L313M, Ara h 1 protein was computationally generated. The region corresponding to the mutant epitope 11 region is shown in white where the substituted residue (methionine) is shown in purple. The remaining atoms of both the wild type and mutant peptide are not shown for clarity. This mutation is not predicted to alter the structure of the mutant protein substantially.

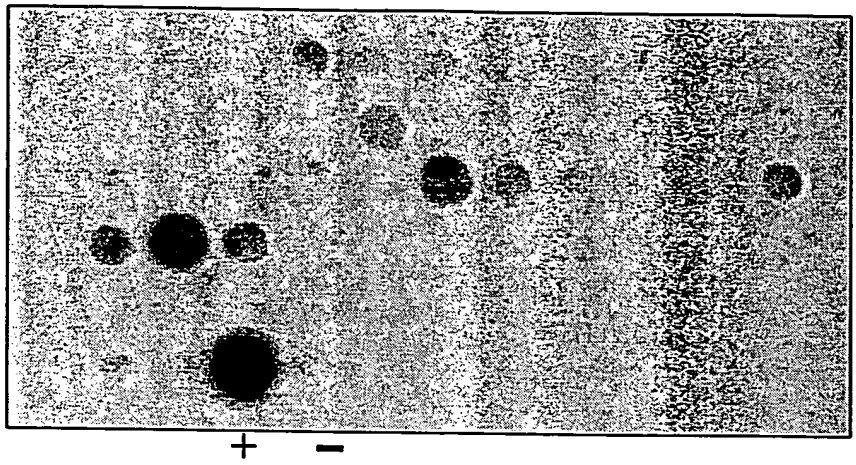
FIGURE 50 Immunoblot of purified recombinant Ara h 3 with serum IgE from individual patients

A

Ara h 3

Amino Acid

1-103
97-199
193-295
289-391
385-487
481-510



B

R1

ISFRQQPEEN ACQFQRLNAQ RPDNRIESEG GYTETWNPNN QEFECAGVAL 50

SRLVLRRNAL RRPFYSNAPQ EIFIQQGRGY FGLIFPGCPR HYEEPHTQGR 100

R2

RSQSQRPPRR LQGEDQSQQQ RDSHQKVHRF DEGDLIAVPI GVAFWLYNDH 150

DTDVVAVSLT DTNNNDNQLD QFPRRFNLAG NTEQEFLRYQ QQSRQSRRRS 200

R3

LPYSPYSPQS QPRQEEREFS PRGQHSRRER AGQEEENECG NIESGFTPEF 250

R4

LEQAFQVDDR QIVQNLRGET ESEEEGAIVT VRGGLRLLSP DRKRRADEEE 300

EYDEDEYEYD EEDRRRGRGS RGRGNGIEET ICTASAKKNI GRNRSPDIYN 350

PQAGSLKTAN DLNLLILRWL GLSAEYGNLY RNALFVAHYN TNAHSIIYRL 400

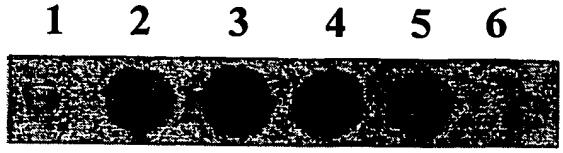
RGRAHVQVVD SNGNRVYDEE LQEGHVLVVP QNFAVAGKSQ SENFEYVAFK 450

TDSRPSIANL AGENSVIDNL PEEVVANSYG LQREQARQLK NNNPFKFFVP 500

PSQQSPRAVA 510

Fig. 51

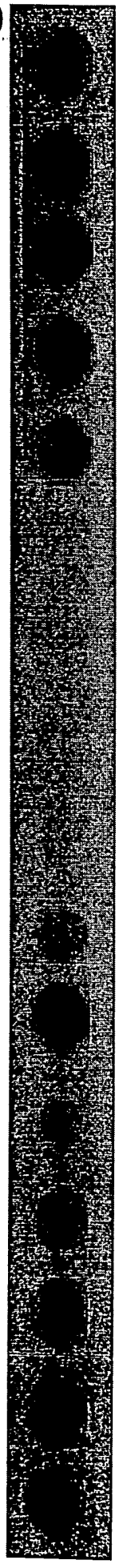
A.



B.

```
    EEEYDEDEYEYDEEDRRRGRGSR
1.  EEEYDEDEYEYDEED
2.    EYDEDEYEYDEEDRR
3.      DEDEYEYDEEDRRRG
4.        DEYEYDEEDRRRGRG
5.          YEYDEEDRRRGRGSR
6.            YDEEDRRRGRGSRGR
```

Fig. 52



D303A

E304A

D305A

E306A

Y307A

E308A

Y309A

D310A

E311A

E312A

D313A

R314A

R315A

R316A

G317A

WT

Fig. 53

FIGURE 54 Recombinant expression and Western blot analysis of the Ara h 3 mutant

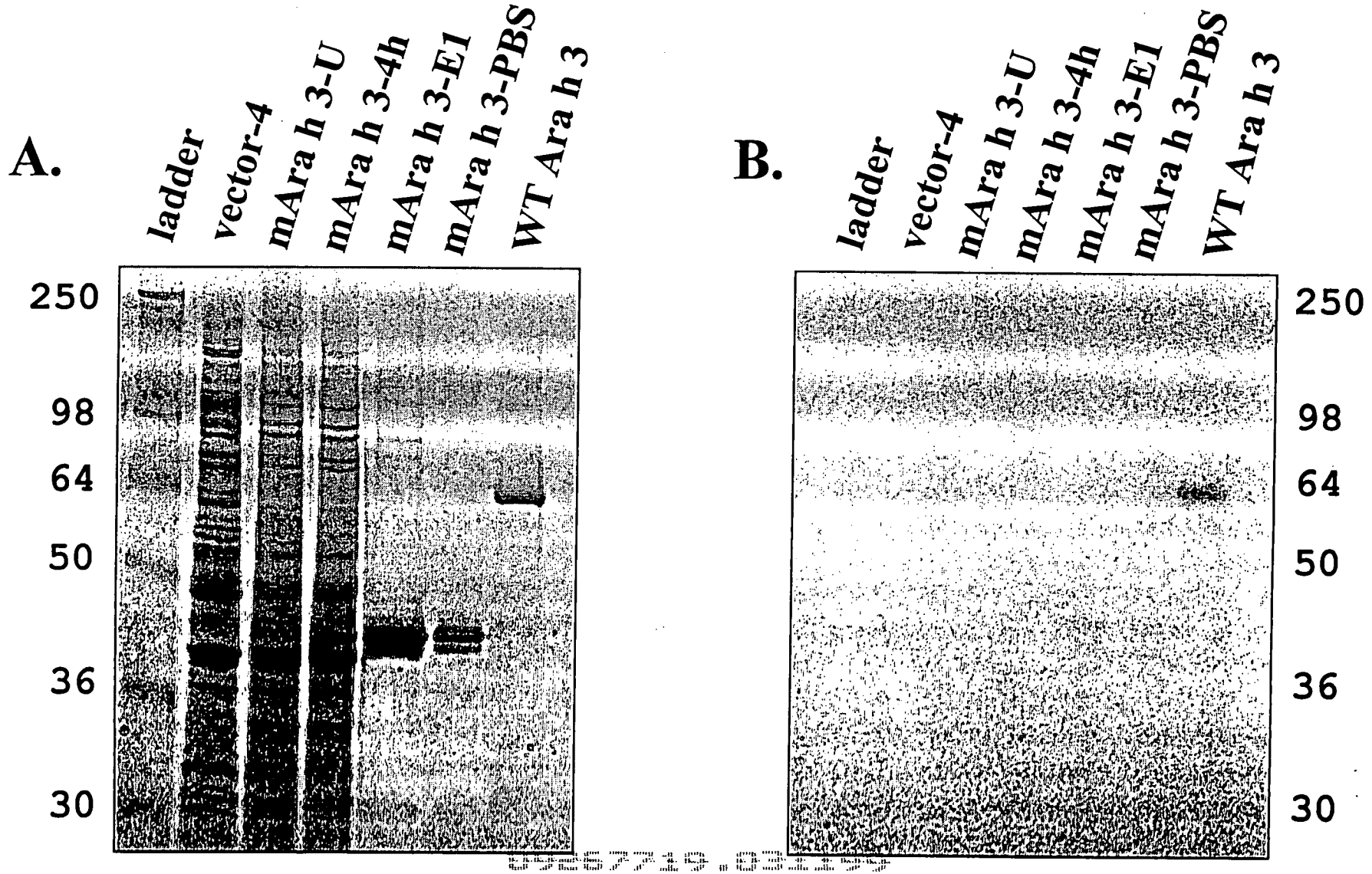


Fig. 55

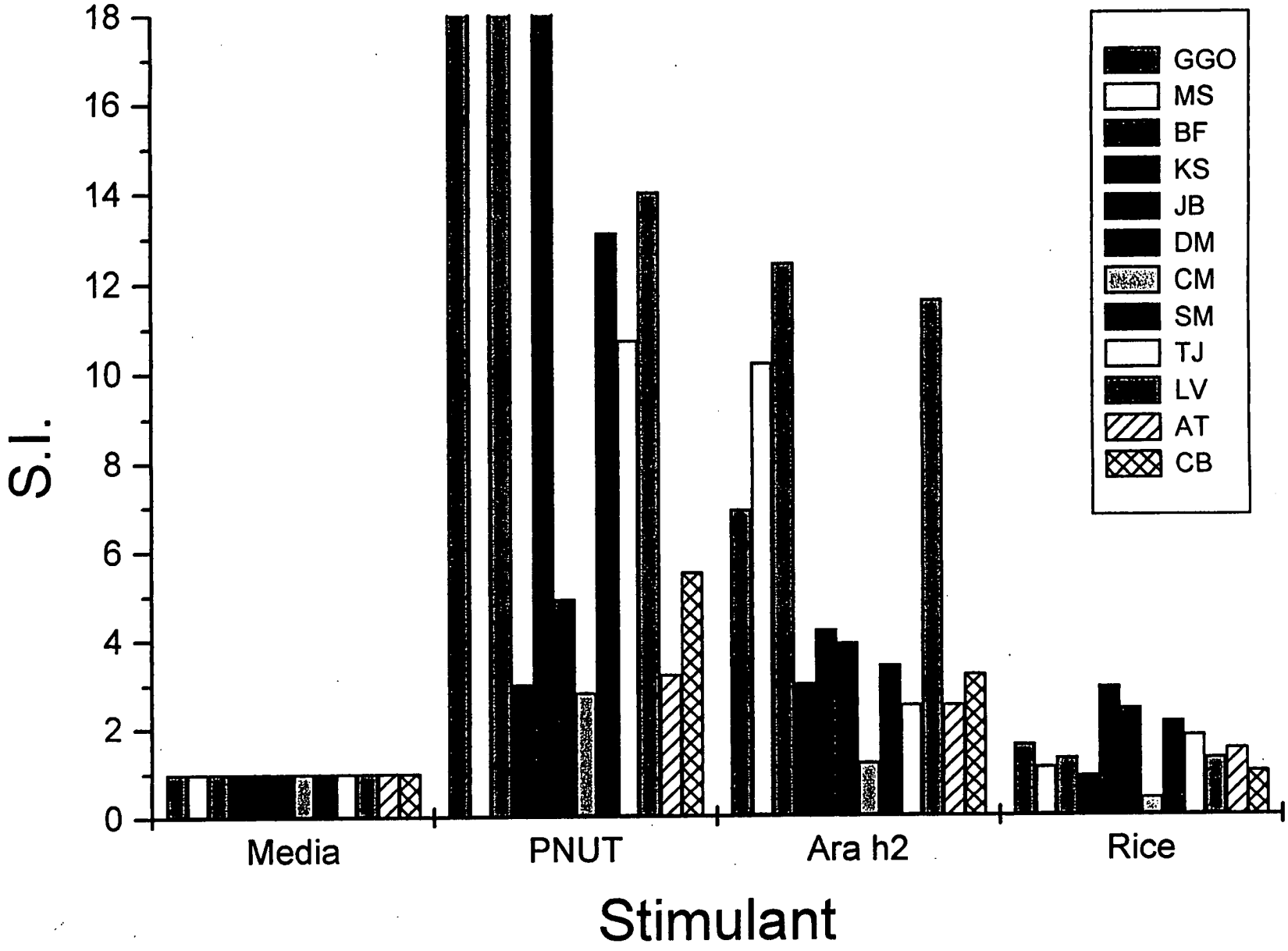
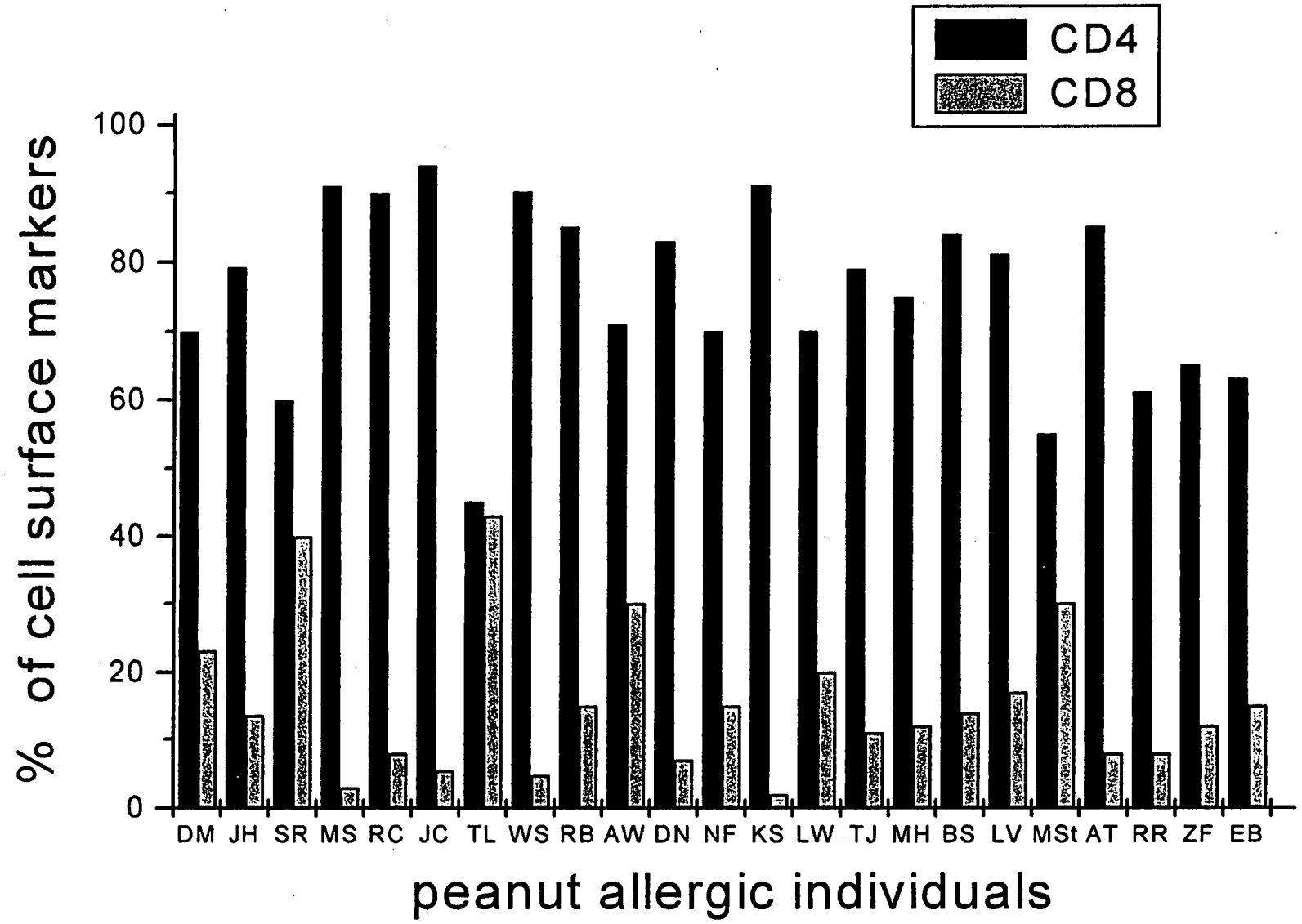

18
16
14
12
10
8
6
4
2
0
S.I.
GGO
MS
BF
KS
JB
DM
CM
SM
TJ
LV
AT
CB
Media
PNUT
Ara h2
Rice
Stimulant

Fig. 56


CD4
CD8
% of cell surface markers
100
80
60
40
20
0
DM JH SR MS RC JC TL WS RB AW DN NF KS LW TJ MH BS LV MSt AT RR ZF EB
peanut allergic individuals